

Salinity:

Environment - Plants - Molecules

Edited by

André Läuchli and Ulrich Lüttge

Kluwer Academic Publishers

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INTRODUCTION

In biology, the very big global and the very small molecular issues currently appear to be in the limelight of public interest and research funding policies. They are in danger of drifting apart from each other. They apply very coarse and very fine scaling, respectively, but coherence is lost when the various intermediate levels of different scales are neglected.

Regarding SALINITY we are clearly dealing with a global problem, which due to progressing salinization of arable land is of vital interest for society. Explanations and basic understanding as well as solutions and remedies may finally lie at the molecular level. It is a general approach in science to look for understanding of any system under study at the next finer (or "lower") level of scaling. This in itself shows that we need a whole ladder of levels with increasingly finer steps from the global impact to the molecular bases of SALINITY relations. It is in this vein that the 22 chapters of this book aim at providing an integrated view of SALINITY.

We have assembled the chapters in a way proceeding from the coarser scales of the very big to the finer scales of the very small. Thus, we start the book with (A) ENVIRONMENT, i.e., the consideration of global impact, the soil and terrestrial ecosystems. From the ecosystem level, we move down to (B) ORGANISMS, i.e., the organism level where we also consider very small and very large organisms (Raven, 1999), such as halophilic prokaryotes and unicellular eukaryotes up to halotolerant higher plants. The reader notices that with the latter sequence we move upwards the ladder from finer to coarser scaling. We continue to do so when we arrange the chapters asking for (C) MECHANISMS starting with membranes and subcellular compartmentation and advancing towards organs and inter-organ correlations to the intact plant. With (D) PHOTOSYNTHESIS, we consider modes of photosynthesis in whole plants, and we begin to move down to the molecular level. This is continued in (E) MOLECULES, where we arrive at the level of molecules, mainly proteins, nucleotides, and lipids.

SALINITY responses of plants constitute an ecophysiological problem, and where we bring it down to the very basis, these responses are a problem of molecular ecophysiology. It must be and effectively is the essential aim of ecology, ecophysiology and molecular ecophysiology to bridge the gap between the very big and the very small and to go through all required steps. Problems encountered globally and in ecosystems must be studied on all the finer levels. This often has been called reductionism, which is, however, a misconception (Lüttge, 1996). We may talk of reductionism only when we reduce complexity. This we do, if within a given level we reduce the number of degrees of freedom of a system considered, using a small selection of essential parts for study. Notwithstanding various attempts applying information theory (Gell-Mann, 1994), it remains unresolved how we might quantify complexity. Ebeling et al. (1998) argue that emergency always leads to increased complexity. However, at least in biology, emergency also implies a change of scale. It appears to be clear intuitively that we do not reduce complexity when we move down scalar levels; for example, molecular biology is no less complex than whole-plant physiology or ecosystem biology. Conversely, of course, it is also a mistake, which is frequently made in our era of

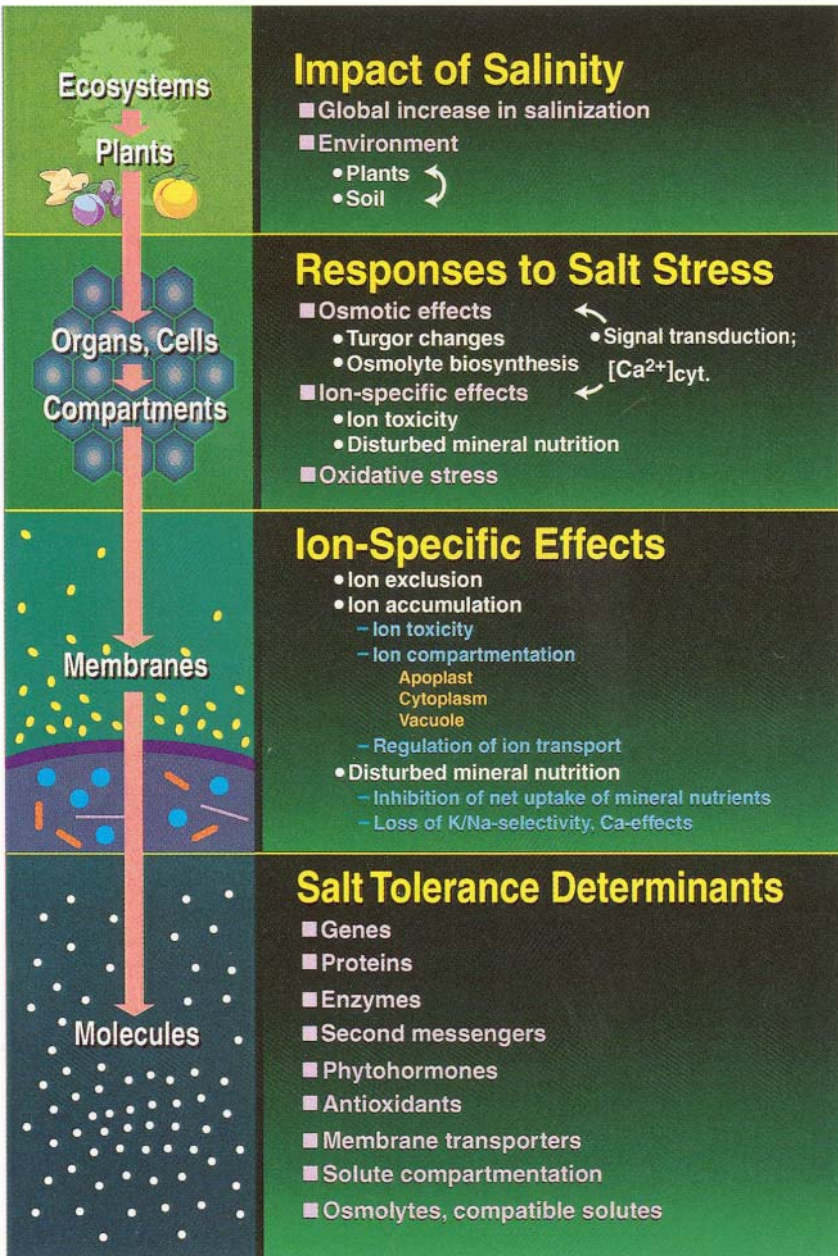
molecular biology, to take it as the goal to arrive at the molecular level and then stay there. Molecular biology itself is now giving us tools for molecular engineering, which allows us climbing upwards to higher levels up to the ecosystem level, where we can use transgenics to test if the molecules we suspected to be essential in SALINITY tolerance really do the job for the plants in their environment. If applied sensibly such approaches of "ecotypic engineering" (Osmond, 1995) open a brilliant future for molecular ecophysiology.

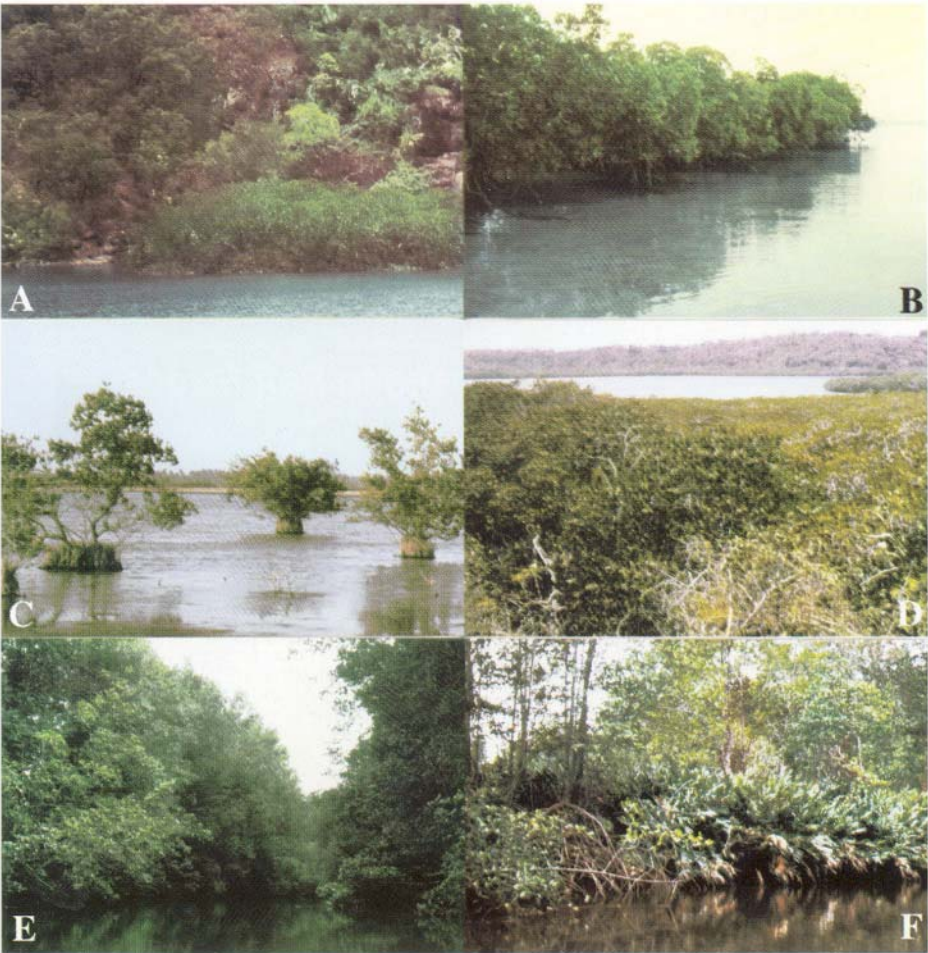
The editors, and with the thrive of all authors to cover general background as well as the current frontiers of research, all participants in this endeavor have done their best to present SALINITY, its current appeal and future challenge, to provide comprehensiveness and stimulation. This may be only a humble attempt in view of many open questions and future progress of research. However, beyond SALINITY, it may also be considered more generally an illustration of how the required integrated approach of molecular ecophysiology can work.

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COLOR SECTION





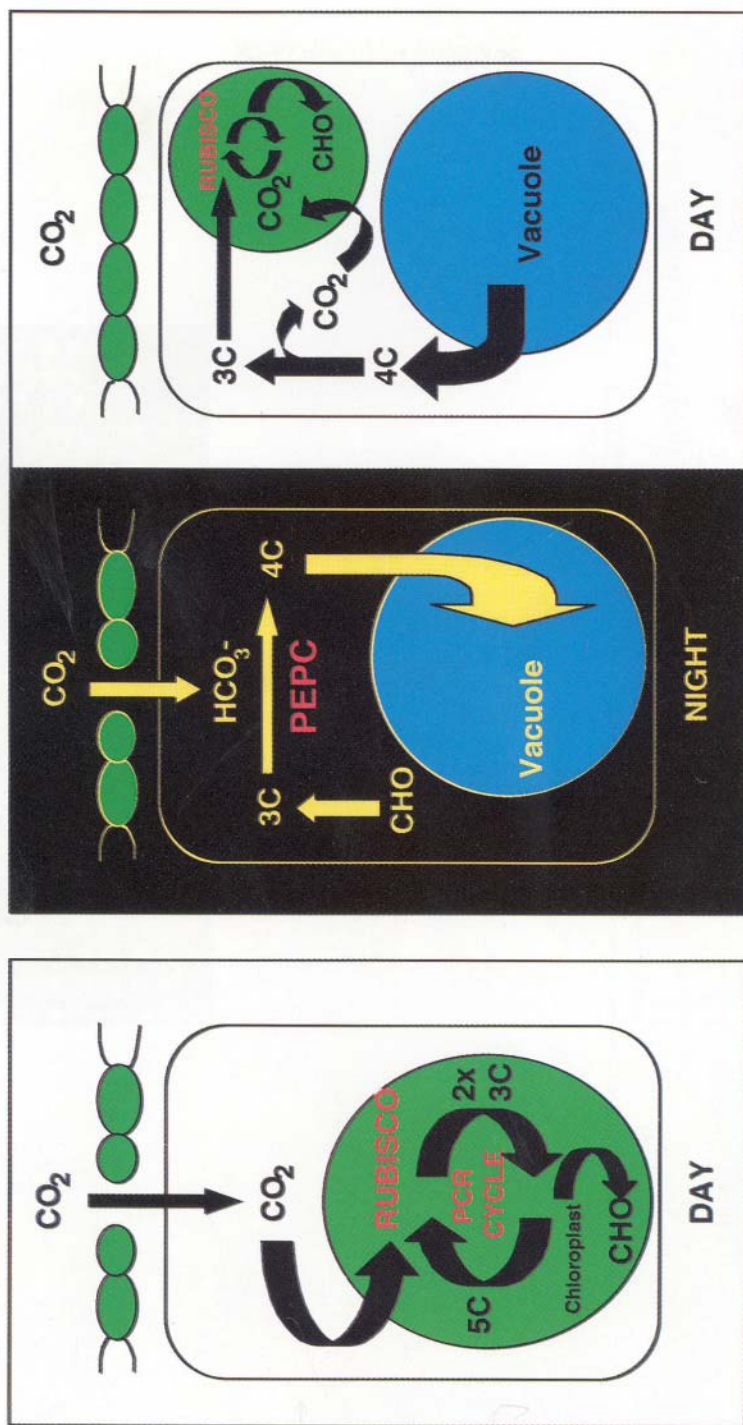
Chapter 6 (U. Lüttge), *Figure 1*, p. 114.



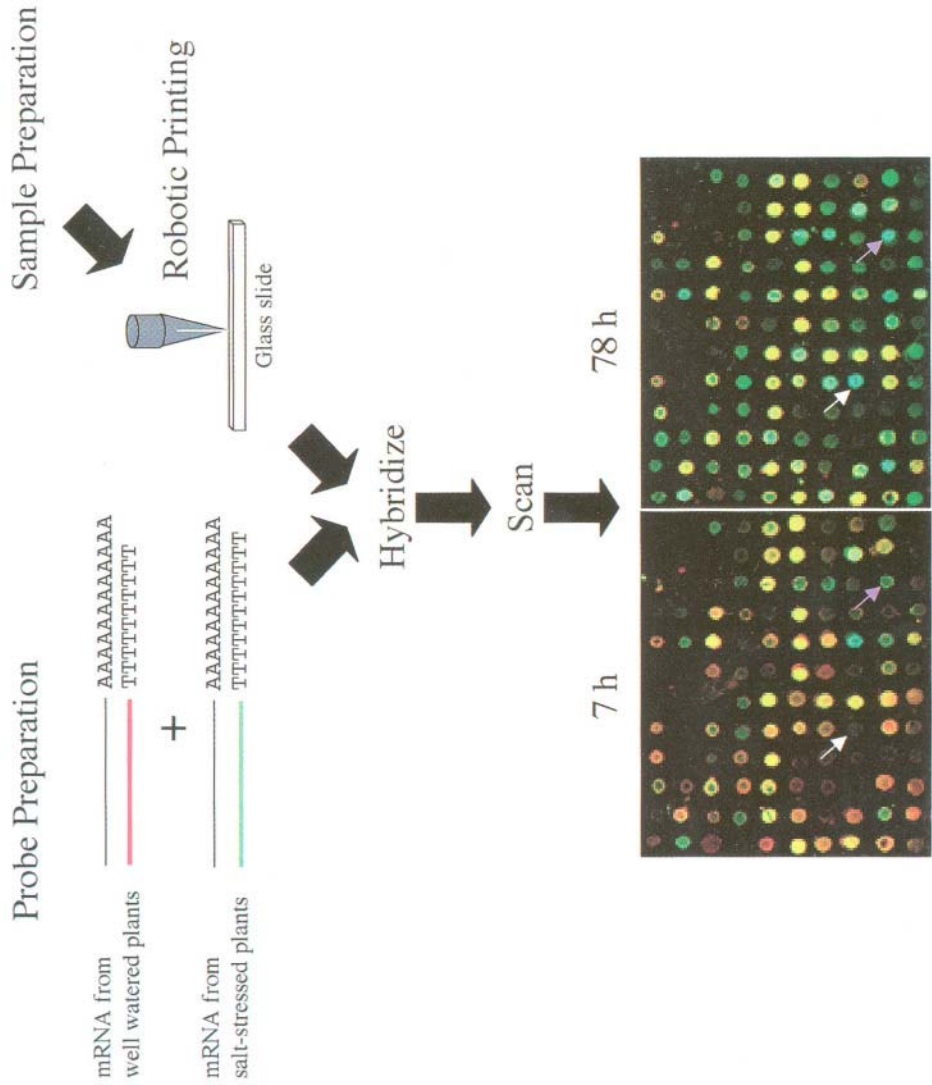
Chapter 6 (U. Lüttge), *Figure 2*, p. 116.

CRASSULACEAN ACID METABOLISM

C3 PHOTOSYNTHESIS



Chapter 17 (J.C. Cushman and H.J. Bohnert), *Figure 1*, p. 363.

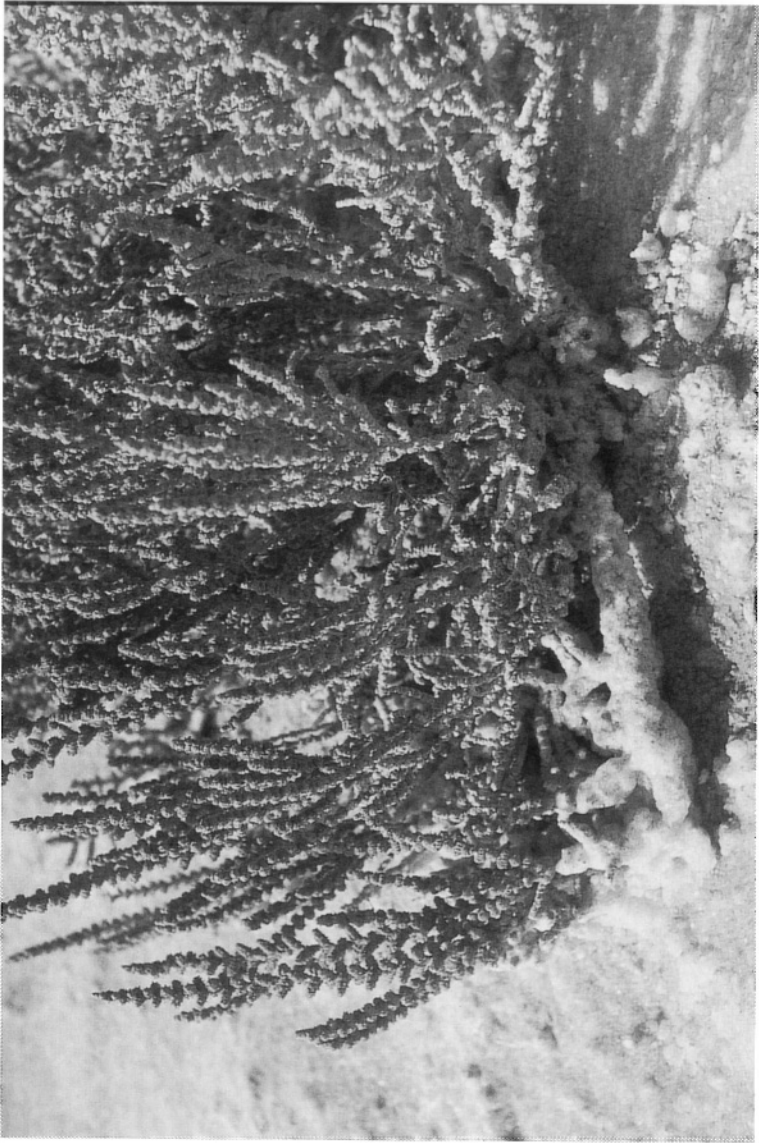


Chapter 17 (J.C. Cushman and H.J. Bohnert), *Figure 1*, p. 374.

A. ENVIRONMENT

Salinity is a global problem. The continuous loss of arable land due to irrigation in arid and semiarid regions of the world, overexploitation and mismanagement contribute to global change in a way which currently appears to draw much less concern in the media and the general public than the accumulation of greenhouse gases - carbon dioxide, methane, and others - in our atmosphere and the putative temperature increase and climate changes associated with them. Nevertheless, the advancement of desertification by salinization and its threatening of global agriculture can be readily quantified. Water is going to be the most cherished resource in this new century, and pessimistic views predict that wars may be fought over it. We have far advanced from the local runoff farming practiced by the Nabateans in Palestine and up to the 12th century, which was successfully reconstructed by Michael Evenari in the Negev of Israel. It is still practiced by some of the semi-nomads in North Africa (e.g., the Djessur-Nomads in Tunisia), and largely avoids irrigation-dependent soil salinization. In this vein, Chapter 1, is setting the scene providing the motivation for and showing the necessity of work on SALINITY.

Although salt spray in wetlands and coastal areas may cause stress by salinity via the atmosphere, salinity typically is an edaphic stress factor as highlighted by our picture of *Halocnemum strobilaceum* sitting in a salt crust on the Schott Fedajj in Tunisia. Hence, we need to understand soil salinity (Chapter 2). Natural saline ecosystems then tell us much about types of environments where diverse life forms of plants cope with salinity (Chapter 3). This allows both to define eco-physiological problems for study at finer levels of scaling and projects on opportunities and limitations of agro-ecosystems management.



CHAPTER 1

GLOBAL IMPACT OF SALINITY AND AGRICULTURAL ECOSYSTEMS

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Abstract

Agricultural losses caused by salinity are difficult to assess but estimated to be substantial and expected to increase with time. Secondary salinization of agricultural lands is particularly widespread in arid and semiarid environments where crop production requires irrigation schemes. At least 20% of all irrigated lands are salt-affected, with some estimates being as high as 50%. Whereas the world's population continues to rise, the total land area under irrigation appears to have leveled off. The need for increased food production therefore needs to be met by increases in yield per land area. To reach this goal, genetic engineering of crop plants for enhanced salt tolerance will be a very important approach. In dry regions where fresh water becomes a scarce commodity, irrigation of moderately salt tolerant crops with brackish water is feasible. Transgenic lines of some crop species have been generated which can grow and develop at fairly high salinity levels in controlled environments. These transgenics must be tested vigorously for yield potential under field conditions.

1.1 Introduction

Salinity is one of the most severe environmental factors limiting the productivity of agricultural crops. Most crops are sensitive to salinity caused by high concentrations of salts in the soil. The cost of salinity to agriculture is estimated conservatively to be about \$US 12 billion a year, and is expected to increase as soils are further affected (Gnassemi *et al.*, 1995). In addition to this enormous financial cost of production there are other serious impacts of salinity on infrastructure, water supplies, and on social structure and stability of communities. Responses to salinization have been of two general kinds; engineering the environment to manage increased salt in the soil by

[†] Michael Pitman died March 30, 2000

irrigation and drainage management, or by “engineering” the plants to increase their salt tolerance. Salt tolerant plants may also ameliorate the environment by lowering the water table in salt affected soils.

This book is mainly about the contribution that scientific research has made to understanding basic mechanisms of salt tolerance of plants and progress made to developing salt tolerance in useful agricultural crops in order to make better use of salt affected soils. That it might be possible to increase salt tolerance of agriculturally useful plants is encouraged by the ability of many flowering plants to live in saline conditions at low water potentials. Mangroves have evolved the ability to grow – and may even require – high levels of salinity. Salt marsh plants grow with periodic inundation of seawater. Certain plants of the seashore, and coral islands (e.g. *Wedelia biflora*) have the ability to grow in wide ranges of salinity and water potential caused by periodic droughts as well as salt stress. Certain semi-arid or arid species (e.g. *Atriplex* spp) are able to tolerate and use salt as an osmoticum, facilitating water extraction from the very low osmotic potentials of desert soils. Within agricultural species there are evident differences in salt tolerance between species and between varieties (Chapter 2, Table 9; Maas, 1990). In 1980 Emanuel Epstein wrote: “The principal responses of halophytes and other plants that tolerate saline environments are of necessity those that lead to osmotic adjustment. Unless that premier adaptation is accomplished existence in saline substrates is impossible.” This is a useful reminder that salt tolerance involves interaction of many processes in the functioning system of the plant and in the ecosystem.

1.2 Global impact of salinization

Salinization commonly occurs as an outcome of agricultural practices, either associated with irrigation or due to long-term changes in water flow in the landscape that can follow land clearance or changed water management. Salinization associated with agriculture occurs when salts build up in the root zone, either because the soil is intrinsically saline, or because the drainage of water from the sub-soil is not sufficient to prevent saline waters rising into the root zone. It therefore tends to be common in arid and semi arid regions where leaching of salt is poor due to low rainfall; where there are strongly saline sub-soils formed from marine deposits or where irrigation changes water tables and salt flow. The ionic balance differs according to the salts in the sub-soils or water. However, Salinization also can be a part of natural landscapes and saline pockets may occur where poor drainage and high soil salinity come together. In these areas salt tolerant species have become established that may have other uses. For example, the salt tolerant grass species *Diplachne fusca*, used as cut fodder in Pakistan, grows naturally in subtropical saline areas in Australia from where it is believed to have been introduced to Pakistan.

In dryland salinity there is the possibility to lower saline water tables by planting trees in the landscape, using salt tolerant trees or deep-rooting shrubs. This possibility has also been considered where irrigation caused secondary salinization of the water table. Such a study was conducted on the western side of the San Joaquin Valley of California in the mid-to-late 1980's, where eucalyptus trees were planted with the intent to lower

saline water tables. The results indicated that although eucalyptus could grow and extract water from the soil under saline conditions, the tree's efficiency in water extraction and thus in lowering the saline water table was curtailed by salinity-induced decrease in evapotranspiration (Dong *et al.*, 1992).

Secondary salinization of arable lands and water resources is as old as the history of human settlement and irrigation. It is a 6,000 year-old problem! History teaches us that between 4000 and 2000 BC the Sumerians ruined their land and in turn their culture in the valleys of the Euphrates and Tigris in Mesopotamia by their irrigation practices, which caused secondary salinization and first eliminated the production of wheat and subsequently that of the more salt-tolerant barley. This led to the demise of the civilizations of Mesopotamia and the rise of Babylon (Jacobsen and Adams, 1958; Boyden, 1987; Läuchli, 1991; Ghassemi *et al.*, 1995). History has repeated itself in this century in many countries. The vast river systems in semi-arid regions with extensive irrigated agriculture not only supply the water for irrigation, but also the salts dissolved in it. These river systems are therefore major contributors to salinity, which plagues these regions.

The scale of the problem of salinization is considerable and continuing to grow. An excellent account of salinization around the world is given in the book "Salinisation of Land and Water Resources" by F. Ghassemi, A.J. Jakeman and H.A. Nix (1995). Climatic groupings are a useful indicator of the nature and potential for salinization (Ghassemi *et al.*, 1995), but functionally it is useful to consider *dryland* or *irrigated* salinity. Dryland (or non-irrigated) salinization is commonly due to rising water tables over essentially saline sub-soils due to clearance of trees or other deep-rooted species, usually to make land available for rain-fed agriculture. It leads to formation of areas with saline seepage instead of fresh water run-off or springs, and is well documented in the history of land clearance in Australia.

Irrigated salinization is the increase in salt in the groundwater that reflects the change in balance between inputs of water and salts, and water and salt drainage. It usually is associated with waterlogging and can lead to saline seepage into low-lying areas. An increased water table can arise from the water distribution system as well as from water application to plants, as has occurred in Pakistan where it is claimed that the ambitious network of irrigation canals built during British rule were unlined and thus leaked to the groundwater.

About 17% of the world's cropland is under irrigation, but irrigated agriculture contributes well over 30% of the total agricultural production (Hillel, 2000). Thus, secondary salinization of irrigated lands is of major concern for global food production. Current estimates indicate that at least 20% of the irrigated lands are salt-affected (Chapter 2; Ghassemi *et al.*, 1995). Other estimates are considerably higher and indicate that up to 50% of all irrigated lands may be salt-affected (Szabolcs, 1992; Flowers, 1999). The coincidence of irrigation and salinization threatens the sustainability of high agricultural productivity (Flowers and Yeo, 1995). In addition, irrigation is not the only reason for salinization of land, as the risk of seawater incursions can lead to tidal intrusion of saline water into rivers and aquifers in coastal areas (Flowers, 1999).

The area of land under irrigation in the world has grown substantially, particularly in the second half of the last century (Figure 1), but appears to have reached limits set by

availability of land and water, cost of managing salt in the water table or pricing policies for water that make irrigated crops less competitive. Available data indicate that total irrigated land area leveled off at about 230 Mha by 1990 and may not increase in the future (Framji *et al.*, 1981; FAO, 1989; Ghassemi *et al.*, 1995). Hillel (2000) confirmed that the total area under irrigation hardly expanded for some years, even as substantial investments have been made in the development of new irrigation projects. He emphasized the reason for this is primarily that large tracts of irrigated land have degenerated due to secondary salinization to the point of being rendered uneconomic to be cultivated. In contrast to the development of irrigated land area over time, leveling off in about 1990, world population continues to increase (Figure 1). The total world population is presently estimated to be about 6.3 billion; it may reach 8.3 billion in 2030 and about 9 billion by 2050. Recent projections by the World Bank (McCalla, 2000) suggest that most of the additional 2 billion people in the next 25-30 years will live in the tropics and subtropics, where the necessary increase in food production cannot be met by increased availability of land and water for irrigation. With no increase in irrigated land area projected in these regions of the world, the need for increased food production to avoid malnutrition and widespread starvation must essentially be met by increased yields per land area (see section 1.5.).

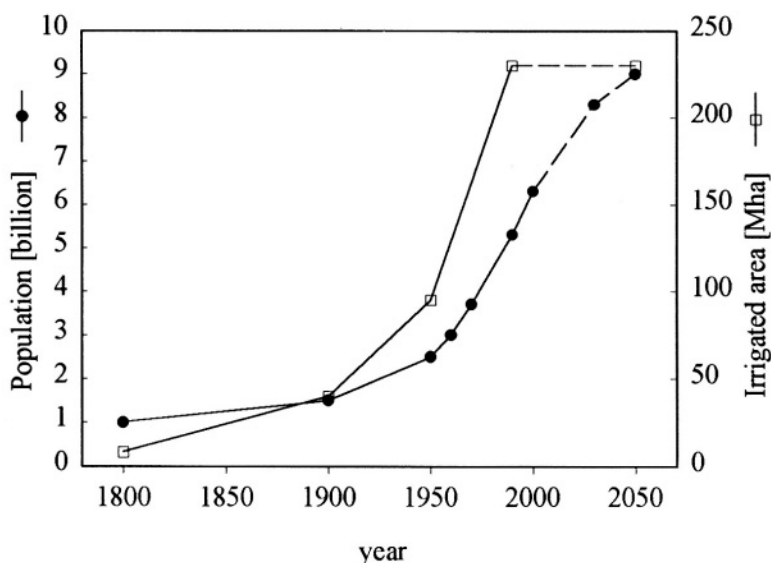


Figure 1. Increase in world population and irrigated land area

All continents of the world have substantial problems with salinization, as shown in maps of the entire world (Tanji, 1990, Figure 1.2) and the USA (Figure 2). In the USA, the dotted areas of Figure 2 indicate that the primary salt-affected regions are located in the western half of this country. The salt-affected agricultural areas in this region are mainly located in California (primarily in the Central Valley and the irrigated Valleys of Southern California), Arizona, North and South Dakota and the coastal regions of South Texas (agricultural and non-agricultural lands are not differentiated in this map).

Salinity-Influenced Soils on Non-Federal Land, 1992

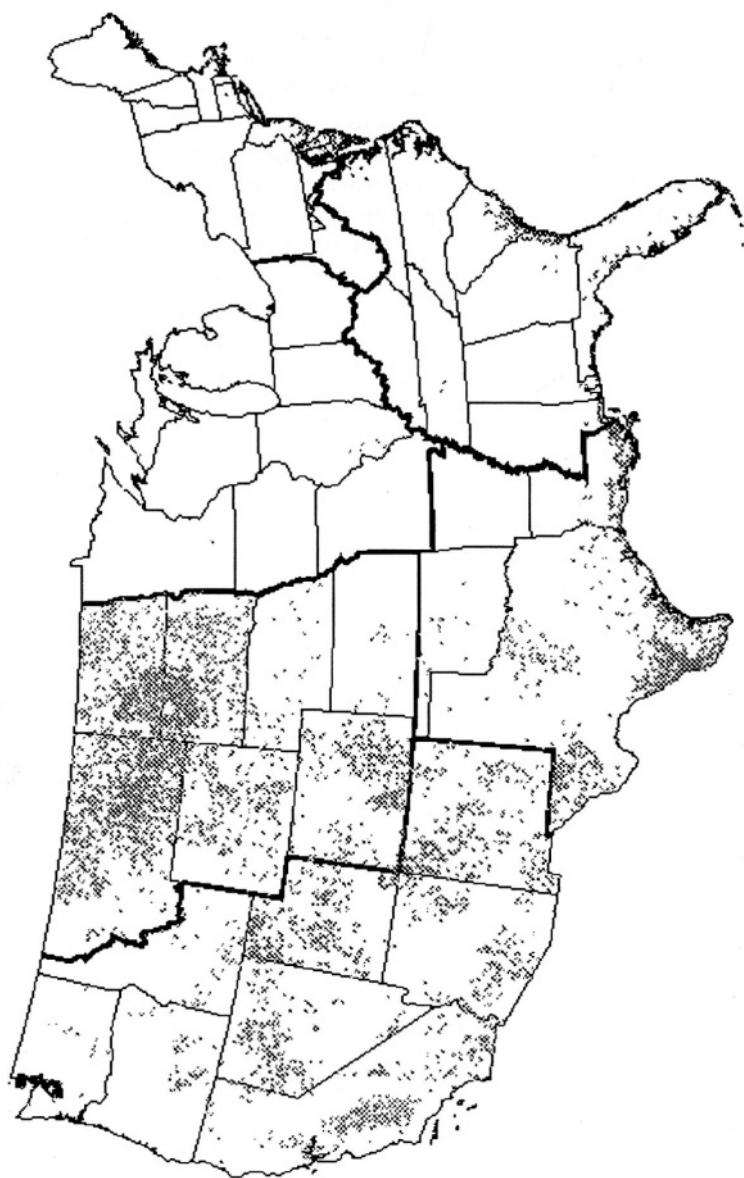


Figure 2. Salinity-influenced soils on non-federal land in the USA in 1992. This dot map shows soils considered "salinity-influenced". One dot equals 10,000 acres of salt-affected land. "Salinity-influenced" soil is where the maximum soil salinity measurement is greater than $EC\ 4\ dS\ m^{-1}$ in the upper 100 cm of soil (from USDA, 1992).

Salinization is widespread in irrigated agriculture (Table 1), particularly in countries of Asia and Africa, with substantial land areas affected in India and China and large proportions of the irrigated land affected in Argentina, Egypt, Iran, Pakistan and USA. The impact of salinity is most serious in countries where all or most of agricultural production is based on irrigation (Egypt, Pakistan) and when agriculture is a substantial part of the national economy. In Australia salinization related to irrigated agriculture is more local in the Murray-Darling catchment, but low irrigation efficiencies and poor drainage have resulted in large areas with high water tables leading to salinization and threatening the citrus and fruit-tree production (McWilliam, 1986). The data in Table 1 do not include the effects of salinity on non-irrigated agriculture. Ghassemi *et al.* (1995) estimated the total soil area degraded by human-induced salinization to be 76.3 Mha. More detailed data on the regional distribution of salt-affected soils are given by Mashali (1999).

Ghassemi *et al.* (1995) also estimated that the global income loss due to salinity was about \$US 11.4 billion per year in irrigated and \$US 1.2 billion per year in non-irrigated areas (based on data by Dregne *et al.*, 1991). This is likely to be a conservative figure, particularly for effects of dryland salinity. Table 2 presents some recent examples of estimated crop and income losses.

TABLE 1. Secondary salinization of the world's irrigated lands (after Ghassemi *et al.*, 1995)

Country	Cultivated land	Irrigated land	Irrigated area	Secondary salinization in irrigated area	
	Mha	Mha	% of cultivated area	Mha	% of irrigated area
Argentina	35.75	1.72	4.8	0.58	33.7
Egypt	2.69	2.69	100.0	0.88	33.0
Iran	14.83	5.74	38.7	1.72	30.0
Pakistan	20.76	16.08	77.5	4.22	26.2
USA	189.91	18.10	9.5	4.16	23.0
Commonwealth of Independent States	232.57	20.48	8.8	3.70	18.1
India	168.99	42.10	24.9	7.00	16.6
China	96.97	44.83	46.2	6.70	15.0
Australia	47.11	1.83	3.9	0.16	8.7
World	1473.0	227.11	15.4	45.4	20.0

The relative loss of production may seem small on a global scale, but where it develops it can have large local impact. In countries where staple crops are produced under irrigation, loss of land and loss of production means less food available locally. Where irrigation is used for higher value crops, financial losses due to salinity or costs of salt management can seriously erode the small margins on agricultural crops. Salinity can thus be another strain on rural communities and contribute to their erosion or to rural poverty.

There are other impacts of salinity, particularly when rising water tables and salinity affect the salt levels in rivers that are sources of water for human consumption and industrial uses.

A recent Australian report (PMSEIC, 1999) estimated that the loss of production due to salinity and rising water tables was about \$US 84 million per year and the capital loss of land was about \$US 450 million. In addition farmers face costs of preventative land management. Rising salt levels occur in many streams and rivers in the south of the continent, leading to environmental degradation (loss of species) estimated at \$US 26 million and a cost to water treatment of about \$US 40 million per year. Damage to

TABLE 2. Selected examples of estimated crop and income losses caused by secondary salinization (estimates for the 1980's and 1990's from several sources)

Country	Region	Secondary salinization % of irri- gated area	Crop loss %	Income loss	
				%	Million US\$ per year
Australia	Murray-Darling Basin				~200
Egypt		33	30		
Israel	Negev			5-45	
Pakistan	Punjab				~300
USA	San Joaquin Valley	39	10		~30
	Colorado River Basin	41-66			~750
Uzbekistan		60	30		
Turkmenistan		80	40		
World					~11,400

infrastructure such as roads, rail and pipelines was put at \$US 65 million per year. There was also widespread damage to foundations of buildings. Thus overall the other effects of salinity could cost as much as the loss in agricultural production and probably more. This report estimated that the area currently affected by salt was about 2.5 Mha but at equilibrium when water tables stabilized the area affected could rise to 15 Mha with associated costs to the community.

1.3 Use of saline water for crop irrigation

Our global water reserves consist primarily of saline waters, i.e., 96.5% is seawater and almost 1% is saline groundwater. This leaves only 2.5% of the global water reserves as fresh, non-saline water, of which two-thirds occurs as ice and only about one-third is fluid fresh-water (Table 3). Thus, there is a limited amount of directly usable fresh water, contrasting with continuing increases in the world population and demand for fresh water. It is estimated that irrigated agriculture presently uses about 65% of the consumed water. However, the extent of water dedicated to irrigated agriculture is likely to be challenged, as pressure is mounting to meet increased demands for human consumption and industrial uses.

The scarcity of fresh water in arid and semi-arid countries poses an additional challenge to irrigated agriculture in these countries. World Bank estimates (1996) for developing dry countries quoted in the special water issue of ICARDA Caravan (1999) suggest "that by 2025 about 3 billion people in 52 countries will face either periodic shortages

TABLE 3. Global water reserves (after Ghassemi *et al.*, 1995)

Source	Volume (Mkm ³)	% of total
Global water	1386	100
Seawater	1338	96.5
Saline groundwater	12.9	0.93
Total saline water	1350.9	97.43
Ice	24.4	1.76
Fresh groundwater	10.6	0.77
Rainfall	0.108	0.008
Total fresh water	35.108	2.538

of water or a chronic scarcity.” This clearly raises questions about the long-term sustainability of irrigated agriculture in dry regions of the world. Adel El-Beltagy, Director General of the International Center for Agricultural Research in the Dry Areas (ICARDA) stated in 1999, “the fresh water currently available per capita in the West Asia and North Africa region is only 1045 m³ as against 3568 m³ in Western Europe and 9529 m³ in North America.” “Non-conventional sources of water will have to be tapped to meet the needs of agriculture in dry areas. Hence developing and refining management techniques that allow the use of marginal-quality water, such as that from municipal wastes, brackish water from drainage schemes, etc., will be important.” Irrigated agriculture in the Middle East and North Africa may still use 75% of the available water, but it is steadily losing out to increasing domestic requirements.

Let us then briefly examine the question whether brackish water can be successfully used for crop irrigation in dry regions. Table 4 shows the classification of water quality based on total salt concentration. In irrigated agriculture water quality is commonly expressed in units of electrical conductivity EC (dS m⁻¹). EC values range from < 0.6 for fresh water to 1.5 – 3 for brackish water to about 45 for seawater. If one relates the range of water qualities to crop salt tolerance, Table 4 indicates that even slightly brackish water can affect the production of such salt-sensitive crops as bean and strawberry, and brackish water affects the moderately-sensitive crops corn, rice, potato and alfalfa. On the other hand, irrigation with brackish water may be feasible for salt tolerant crops, such as barley, sugar beet and cotton.

In the San Joaquin Valley of California the disposal of saline drainage water from salt-affected irrigated lands has been a controversial issue, and recycling of such waters has been considered for crop irrigation. A few feasibility studies under field conditions have been conducted and will be summarized. Rhoades *et al.* (1988) did a field study in the Imperial Valley of California, which demonstrated that moderately saline water up to about EC 4 dS m⁻¹ can be used for irrigation of salt tolerant crops provided that some leaching occurred through preplant irrigation with fresh water. After preplant irrigation, acceptable yields for cotton and sugar beet were achieved in the San Joaquin Valley by irrigation with water of an EC of about 9 dS m⁻¹ for three years (Ayars *et al.*, 1986 a, b).

In a related study on a tomato – cotton rotation, using fresh water for irrigation until plants were established and applying saline drainage water with EC ≈ 7.4 dS m⁻¹ for irrigation during two years out of three, yields of tomato and cotton were little affected until the third year of the experiment (Shennan *et al.*, 1995). These field studies, however, were not conducted long-term enough to fully assess the impacts of saline

water irrigation on crop productivity and soil degradation. Such a long-term field study has been completed and its results published by Goyal *et al.* (1999 a, b).

A nine-year crop rotation (cotton – cotton – safflower, 3 times, 1984-1993) field experiment was conducted in which only cotton was irrigated with mixtures of saline drainage water ($EC = 11.6 \text{ dS m}^{-1}$) and fresh water to achieve saline irrigations varying from EC 0.95 to 11.6 dS m^{-1} . The experimental location consisted of an 8 ha field site in the Tulare Lake Basin of the San Joaquin Valley in Central California. The entire site was pre-irrigated with fresh water every year in January. Safflower was grown only with a preplant irrigation with fresh water, but it was indirectly affected by the residual effect of applying saline water for irrigation of cotton. Figure 3A shows cotton lint yield data over six cropping years as affected by salinity of the irrigation water. Detrimental effects were first observed in the third cotton crop, and in the fifth cotton crop, salinity greater than 3000 ppm total dissolved solids ($EC \sim 5 \text{ dS m}^{-1}$) led to a reduction in lint yield. Fiber quality, however, was not affected at all salinity levels. Lint yield reduction was a function of time and salinity level of the irrigation water and appeared to be caused mainly by a reduction in stand establishment. Interestingly, there was some correlation between yield reduction and decrease in K / Na ratios of leaf blades and petioles. Adverse effects of the higher salinity levels on soil structure also impacted stand establishment and yield of cotton. It was concluded that irrigation of cotton with saline water of up to $EC = 5 \text{ dS m}^{-1}$ can be applied for four years without any reduction in yield, provided that soil leaching is achieved through preplant irrigation with fresh water.

Figure 3B shows the safflower seed yield data over three cropping years. Yields were reduced in the first year by a salinity level of $EC = 7 \text{ dS m}^{-1}$ and more severely in the second and third crop. Previous irrigation with water of the highest salinity level ($EC =$

TABLE 4. Classification of water quality (based on total salt concentration) and salt tolerance of selected herbaceous crops (after Hillel, 2000 and Maas, 1990)

Salt tolerance ratings: sensitive (S), moderately sensitive (MS), moderately tolerant (MT), tolerant (T)

Water designation	Total dissolved salts, ppm	EC (dS m^{-1})	crop	threshold EC (dS m^{-1})	salt tolerance rating
Fresh water	< 500	< 0.6			
Slightly brackish	500 - 1,000	0.6 – 1.5	bean	1.0	S
			strawberry	1.0	S
Brackish	1,000 – 2,000	1.5 – 3	corn, potato	1.7	MS
			alfalfa	2.0	MS
			rice	3.0	MS
Moderately saline	2,000 – 5,000	3 – 8	soybean	5.0	MT
			wheat	6.0	MT
			sorghum	6.8	MT
			sugar beet	7.0	T
			cotton	7.7	T
			barley	8.0	T
Saline	5,000 – 10,000	8 – 15			
Highly saline	10,000 – 35,000	15 - 45			

11.6 dS m⁻¹) severely reduced safflower seed yield to 14% of the control in the third crop, but the seed oil content and quality were not affected by salinity.

A general conclusion from this nine-year field study is that the feasibility of crop irrigation with saline water needs to be evaluated over several years, for each specific crop species, and for each soil and climatic environment. Some leaching of the soil should be applied between cropping seasons to control soil salinity, and only irrigation with water of moderate salinity levels appears to provide reasonable yields of moderately tolerant and tolerant crop species. For a more in-depth evaluation of irrigation with saline water, the reader is referred to the review by Shalhevet (1994).

1.4 Scientific progress

Physiological studies have given an insight into how plants handle high salinities and the associated low osmotic potential and identified critical activities at cell membranes or in the cytoplasm. Such studies have been enriched by comparison of the diversity among plants in their adaptations to salinity. Scientific progress would have been poorer if studies had been restricted to a few species. This research has focused on key transport processes and reinforced the importance of understanding the “system” in which processes of water transport, salt and nutrient transport and cytoplasmic activity and homeostasis interact.

Two areas of physiology that have given particular insight into tolerance of salt are compartmentation and osmoregulation in the cytoplasm. Compartmentation here means identification of the different salt strategies of different cells in the plant and of the parts of the cell, particularly the cytoplasm and the vacuole, that result in selective accumulation of sodium and chloride into cell vacuoles and the excretion of salt in glands or hairs. Understanding of the sharp membrane boundaries to ionic distribution has been aided by technologies such as X-ray microanalysis, electrophysiology including patch-clamping and ion-sensitive microelectrodes and intracellular pressure probes.

A significant quantitative approach has contributed considerably to our mechanistic knowledge of the effects of salinity on plant growth and development. The approach pioneered by Wendy Silk (Silk, 1984; Silk *et al.*, 1986) relates quantitative data on the spatial distribution and temporal patterns of root and leaf growth to the spatial and temporal patterns of substance content (mostly inorganic and organic solutes), using a continuity equation. This allowed the calculation of local net deposition rates, for example of ions and organic solutes such as sugars, and gave new insight into the role of mineral nutrient ions and of organic solutes in the effect of salinity on growth and development of plant organs (Zhong and Läuchli, 1994; Bernstein *et al.*, 1995; Hu and Schmidhalter, 1998 a, b). General conclusions from using this approach were that root growth under saline conditions depends on the maintenance of K / Na – selectivity in the growing zone of the root, and that Na accumulation in the growing region of a leaf does not appear to cause ion toxicity and therefore may not be responsible for growth inhibition of the leaf.

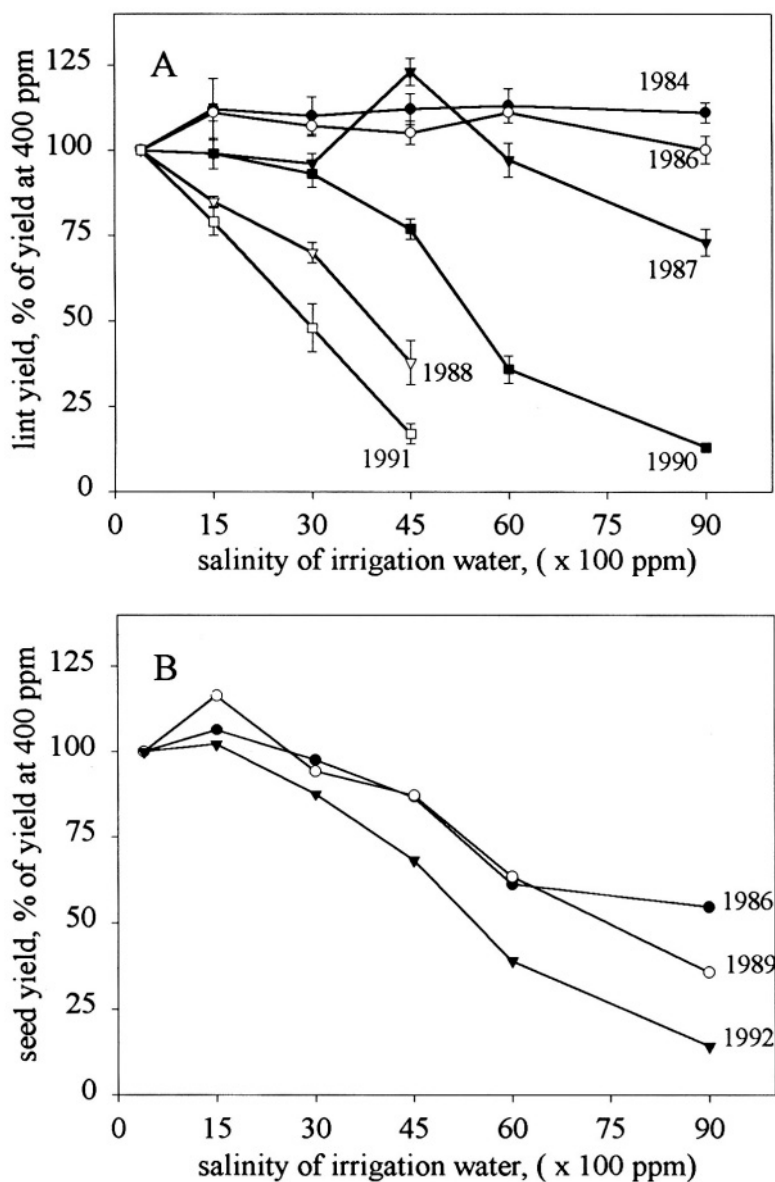


Figure 3. A. Lint yield of field grown cotton as affected by irrigation water quality, expressed as % of yield at 400 ppm ($EC = 0.95 \text{ dS m}^{-1}$) irrigation water quality (control). Data shown over six cropping years (1984-1991) (from Goyal *et al.*, 1999 a).

B. Seed yield of field grown safflower as affected by the residual effect of saline water irrigation of cotton, expressed as % of yield at 400 ppm ($EC = 0.95 \text{ dS m}^{-1}$) irrigation water quality (control). Data shown over three cropping years (1986, 1989, 1992) (from Goyal *et al.*, 1999 b).

As far as osmoregulation in the cytoplasm is concerned, the recognition of organic osmolytes (termed compatible solutes) such as betaines, proline and sugar compounds that can accumulate at high levels in the cytoplasm without any deleterious effect on enzyme processes and proteins is one of those apparently simple discoveries that have disproportionate power of explanation.

The processes involved in compartmentation and osmoregulation in the cytoplasm are critical for the maintenance of cellular ion homeostasis in saline environments (Niu *et al.*, 1995; Serrano *et al.*, 1999; Hasegawa *et al.*, 2000). Salinity causes perturbations of the steady state of sodium, chloride, potassium and calcium ions in the cell. Ion homeostasis can be restored and maintained by transmembrane transport proteins that mediate ion fluxes. Critical components of cellular ion homeostasis under salinity stress are the accumulation of Na and Cl in the vacuole, the maintenance of physiological concentration of K in the cytoplasm, and the regulation of low Ca activity in the cytoplasm. An important consequence of Na and K homeostasis is the cellular maintenance of K/Na – selectivity, and it is now well recognized that maintained K/Na – selectivity is a critical feature of salt tolerance in plants (Läuchli, 1999 a).

Figure 4 presents an overview of the mechanisms of salt tolerance and salt toxicity in higher plants. Salt tolerance mechanisms need to be investigated at several scales, all the way from the ecosystem level through whole-plant responses to membrane processes and down to the molecular level. Traditionally, the responses to salt have been categorized into “osmotic effects” and “ion-specific effects” (Epstein and Rains, 1987; Läuchli and Epstein, 1990). And it has been recognized for some time that perturbation of Ca activity in the cytoplasm caused by salinity stress (Lynch *et al.*, 1989) leads to signal transduction that triggers either acclimation to salt stress or salt toxicity (Liu and Zhu, 1998; Epstein, 1998; Bressan *et al.*, 1998; Hasegawa *et al.*, 2000). In recent years, oxidative stress has also been recognized as an important component of plant response to salt stress (Polle and Rennenberg, 1993; Shalata and Tal, 1998). In-depth treatments of the osmotic and ionic effects of salt stress are presented in several chapters of this book, particularly in the sections C (mechanisms) and E (molecules).

Enormous progress has been made in the last ten years in the molecular and genetic understanding of plant responses to salt stress. Studies in molecular genetics have identified the sequences and structures of transport systems and enzymes responsible for cytoplasmic regulation. Salt tolerance of plants is a multigenic trait. A number of genes have been identified that contribute to the regulation of salt tolerance in plants. Genes that regulate plant selectivity of potassium over sodium appear to play a particularly critical role in salt tolerance and salt sensitivity (Gassman *et al.*, 1996; Zhu *et al.*, 1998; Serrano *et al.*, 1999; Hasegawa *et al.*, 2000). Genes controlling the expression of compatible solutes in the cytoplasm are likely to be of similar significance to salt tolerance (Bohnert and Shen, 1999; McNeil *et al.*, 1999; Holmström *et al.*, 2000; Sakamoto *et al.*, 2000). Through multiple gene transfer (Bohnert *et al.*, 1995), entire metabolic pathways involved in the synthesis of compatible solutes can now be genetically manipulated, for example the biosynthesis of proline (Delauney and Verma, 1993) and glycine betaine (McCue and Hanson, 1990). Recently, Eduardo Blumwald's group identified a family of genes that encode for plant Na^+/H^+ antiporters at the tonoplast and demonstrated their significance in vacuolar sodium accumulation (Apse *et al.*, 1999; see also Blumwald and Gelli, 1997). Sodium sequestration and accumulation

in the vacuole appear to control, by overexpressing AtNHX1, normal growth and development of transgenic *Arabidopsis* (Apse *et al.*, 1999) and tomato and *Brassica* (canola) (Blumwald, 2001) in a culture medium salinized with 200 mM NaCl. The characterization of genes that contribute to salt tolerance and the underlying physiological processes could lead to the identification of specific physiological and biochemical markers for salt tolerance (Läuchli, 1999 b). Such possible markers more broadly termed ‘salt tolerance determinants’, are enlisted at the molecular scale in Figure 4.

Salt tolerance appears to be due to a suite of genes that contribute to salt and osmotic regulation in different parts of the cell and the plant, to maintenance of photosynthesis (see chapters in section D), and to reproduction and seed production under saline conditions. For some time plant breeders and physiologists have worked with this opportunity by screening and selecting plants for performance under saline conditions (Epstein *et al.*, 1980; Epstein, 1985; Shannon and Noble, 1990). Osmond (1995) recently suggested, genetic engineering of plants for salt tolerance should also address “ecotypic engineering” to produce plants with improved salt tolerance that are able to flourish in different environmental conditions. Most researchers have focussed on using NaCl to mimic salinity in the soil environment. However, some salt-affected soils are characterized by sulfate rather than chloride salinity. For example, salinity in Canadian prairie soils is predominately caused by sulfate salts (Curtain *et al.*, 1993). In several crops, however, sulfate salinity causes growth effect different from those by chloride salinity, for example in sorghum (Boursier and Läuchli, 1990). The mechanisms of this differential plant response are largely unknown. Other salt-affected soils can contain elevated concentrations of certain toxic metal elements such as cadmium or high concentrations of boron that are toxic to boron-sensitive plants (Page and Chang, 1990). The possible interactive effects of salinity and metals or boron on plants have not been investigated sufficiently, and the mechanisms of these interactive soil stresses are largely unknown. Since these stress interactions occur particularly in agricultural soils, it is important to also conduct investigations of crop responses to these more complex and adverse soil conditions.

1.5 Outlook

Feeding the growing world population has been a strong moral impetus for agricultural scientists. At different times the problem has seemed insurmountable but at others there has been confidence in the ability to use technology to grow sufficient food. One such period was following the “green revolution”. At present world opinion seems to be in a confident phase, but with concerns about what might happen to global food production in 20 – 50 years, particularly in developing countries.

In recent decades there has been increased recognition that production must be sustainable. Erosion, salt affected land, loss of environmental quality, and urban or infrastructural encroachments on agricultural land reduce the potential for future generations to feed themselves. Looking forward into this century, it is evident that the increasing levels of greenhouse gases will have an effect on global temperatures. However, the well documented increases in atmospheric CO₂ will likely exacerbate the

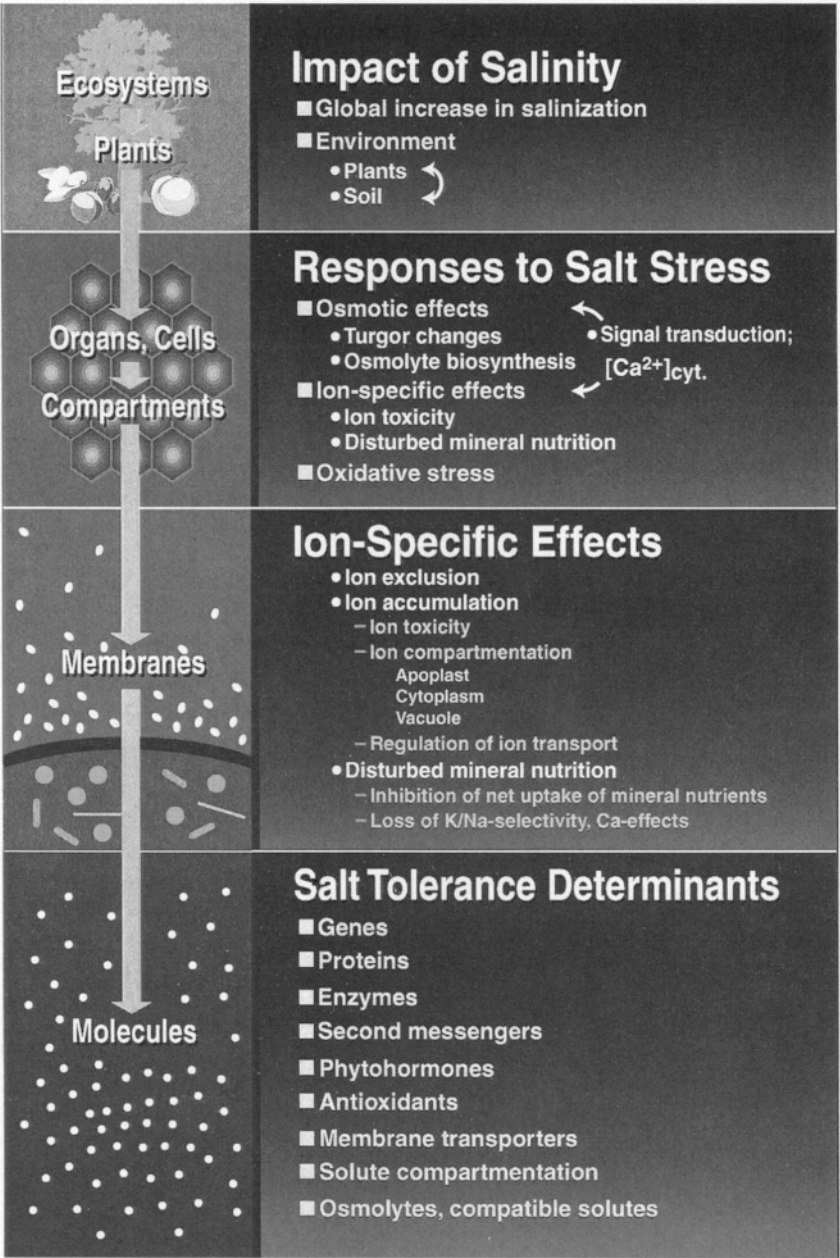


Figure 4. Overview of mechanisms of salt tolerance and salt toxicity in plants
(see color version in the color section)

detrimental effects of salinity on agriculture (Yeo, 1999), rather than partly ameliorate the problem, as has been proposed earlier by some investigators (see also Chapter 15).

We are convinced that plant biotechnology approaches with the aim of producing transgenic crops that show enhanced salt tolerance and performance under field conditions will become an essential component of sustainable crop production on salt-affected lands. In the late 1980's, the future role of biotechnology for production of more salt-tolerant crops was already predicted (Läuchli, 1987). About 15 years later, major breakthroughs in this complex task are now occurring. Although the majority of genetic advances in salt tolerance has been accomplished using the relatively salt-sensitive *Arabidopsis* (Sanders, 2000; Zhu, 2000), recent development of transgenic crops by Blumwald's group (described in section 1.4) are very promising. Moreover, Zhu (2000, 2001) discovered a salt-tolerant relative of *Arabidopsis*, the halophyte *Thellungiella halophila*, which is a plant native to the coastal saline lands of eastern China, survives seawater salinity and completes its life cycle in the presence of 300 mM NaCl (Zhu, 2000). Since *T. halophila* is a close relative of *Arabidopsis* and has > 90% nucleotide identity in cDNA sequences, it may become a very useful model plant species for salt tolerance studies. In addition, genomic approaches will be particularly useful in effective engineering of plants for greater salt tolerance (Cushman and Bohnert, 2000).

Assuming genetic engineering for production of salt tolerant transgenic crops will be successful in the near future on a broad scale, it will provide us with crop plants that show superior productivity on salt-affected soils in comparison with existing varieties and cultivars. Then in many cases the progress most needed is with local solutions that address land management and may require changes in farming practices. In addition, governments need to address policies to encourage suitable water and salt management, for example through policy for water use and pricing or support for infrastructure in order to protect the value of the soil for the future and maintain other society values such as environmental use of water.

The importance of finding local solutions to salinity as a constraint to agriculture should not be taken to mean that there is less need for study of the processes by which plants are able to be productive in saline conditions. Molecular genetics based on understanding of the processes of salt tolerance and its application through genetic engineering is the most likely means of increasing salt tolerance and retaining productivity in a wide range of agriculturally useful species.

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CHAPTER 2

SALINITY IN THE SOIL ENVIRONMENT

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Abstract

The purpose of this chapter is to provide plant scientists with a background on the nature of soil salinity with a particular emphasis on irrigated agriculture. Since the chemistry of soil solutions plays a major role in soil salinity, considerable details on this topic are offered. Chemical speciation in the soil solution should be of importance to plant scientists. The dynamic nature of soil salinity in the rootzone affects performance of plants. Profile distribution of salts is affected by leaching fraction and changes with changing water content from irrigation and rootwater extraction. Soluble salts in soils are highly mobile and transported by water through mass flow and dispersion. Irrigation water management is one of the keys in maintaining salt balance in the rootzone. Growing regulations on the disposal and management of poor quality drainage waters is now exacerbating the maintenance of salt balance in the rootzone in irrigated lands.

2.1 Introduction

The world's surface area occupies about 13.2 billion ha, but no more than 7 billion ha are arable and 1.5 billion ha are cultivated (Massoud, 1981). Of the cultivated lands, about 0.34 billion ha (23%) are saline (salt-affected) and another 0.56 billion ha (37%) are sodic (sodium-affected). Thus, saline and sodic soils cover about 10% of the total arable lands and exist in over 100 countries. Another set of database (FAO, 1989) indicates that the world has about 227 million ha of irrigated lands of which 20% are salt-affected. Since irrigated agriculture provides about one third of the world food supply, secondary salinization of irrigated lands is of major concern. And of the remaining 1,247 million ha of non-irrigated lands, 31.2 million ha are salinized. Ghassemi *et al.* (1995) point out that land degradation, including soil salinization, is a

principal constraint in meeting the needs of world food production. The reader is directed to Chapter 1 for more details.

2.2 Nature of Soil Salinity

2.2.1 SALINITY CONSTITUENTS AND SALINITY PARAMETERS

Salinity as defined herein is the concentration of dissolved mineral salts present in soils (soil solution) and waters. The dissolved mineral salts constitute a mixed electrolyte of cations and anions. The major cations in saline soil solutions consist of Na^+ , Ca^{2+} , Mg^{2+} and K^+ and the major anions, Cl^- , SO_4^{2-} , HCO_3^- , CO_3^{2-} and NO_3^- . Other constituents contributing toward salinity in hypersaline soils and waters include B , Sr^{2+} , SiO_2 , Mo , Ba^{2+} and Al^{3+} . These salinity constituents are reported in units of mmol/L or mmol charge/L (meq/L) or mg/L (ppm) for major solutes and $\mu\text{mol/L}$ or $\mu\text{mol charge/L}$ or $\mu\text{g/L}$ (ppb) for trace elements. Smaller but important activities of H^+ and OH^- are also present and they are respectively reported in terms of pH and pOH.

Salinity in soils and waters is often reported as a lumped parameter, i.e., Electrical Conductivity (EC) or Total Dissolved Solids (TDS), and sometimes, Total Soluble Cations (TSC) and Total Soluble Anions (TSA). EC is an intensive (electrical) parameter and reported as milliSiemens/cm (mS/cm, equivalent to $\mu\text{mhos/cm}$) for lower salinities and deciSiemens/m (dS/m, equivalent to mmhos/cm) for higher salinities. A saline solution offers smaller resistance to the passage of an electric current and hence has higher conductance while a dilute solution offers greater resistance and hence has lower conductivity. TDS is an extensive (capacity or gravimetric) parameter and is reported as mg/L or g/L for hypersaline waters and soils. TDS is obtained by evaporating a sample of water or soil solution down to dryness in which a portion of the carbonates is lost as CO_2 gas during desiccation. TSC and TSA are reported as mmol charge/L or meq/L and are obtained from detailed chemical analyses of cations and anions. TSC and TSA are frequently compared to check on the accuracy of chemical analyses, i.e., salinity is a heterogeneous mix of electrolytes in which the charges (cations and anions) should be balanced assuming that all of the major ions have been analyzed.

No exact relationship exists between intensive and extensive lumped salinity parameters, however, approximate conversion factors are used (Tanji, 1990 a). TDS may be approximated by taking the product of EC in dS/m and 640 for solutions up to about EC 5 dS/m or for more saline waters and soil solutions, EC in dS/m and 800. Taking the product of EC in dS/m and 10 approximates TSC or TSA in meq/L. Taking the product of EC in dS/m and 0.00364 approximates solute potential (osmotic pressure) in MPa. Taking the product of EC in dS/m and 0.0127 approximates ionic strength in M/L.

2.2.2 DYNAMIC NATURE OF SOIL SALINITY

The measurement of salinity in waters for EC, TDS, TSC and TSA is straightforward. In contrast, the measurement of soil salinity is challenging because of the strong influence

of soil moisture content. The concentration of dissolved mineral salts generally does not change in direct proportion to changes in soil water contents due to mineral solubility, cation exchange and ion association (Tanji *et al.*, 1967). Thus, salinity is a dynamic property in the actively transpiring plant root zone due, on one hand, to evapoconcentration of the soil solution from root-water extraction and evaporation from moist soil surfaces and, on the other hand, to replenishment of soil water from rainfall, snowmelt or irrigation. Moreover, the dissolved mineral salts are highly mobile in the soil profile due to chemical diffusion (ion mobility) and the convective and dispersive transport by soil water. Therefore, the plant roots are exposed to dynamic temporal and spatial changes in soil salinity and they pose a challenge in measuring soil salinity.

Electrical conductivity is measured in the extract of soil samples saturated to a reference water content (ECe) or in soil solutions extracted by vacuum with suction cups installed in the soil (ECsw) under moist conditions or by in situ electroconductivity technique (ECa for apparent bulk EC) such as the Wenner array of electrodes, four-electrode salinity probe or an electromagnetic device (e.g., EM-38 probe). Some claim the Time Domain Reflectometry (TDR) device has the potential to measure salinity, too.

Figure 1 (Hoffman *et al.*, 1983) shows the salinity measured in sprinkled and subsurface irrigated soils of corn (*Zea mays*) plots using saturated soil samples (ECe),

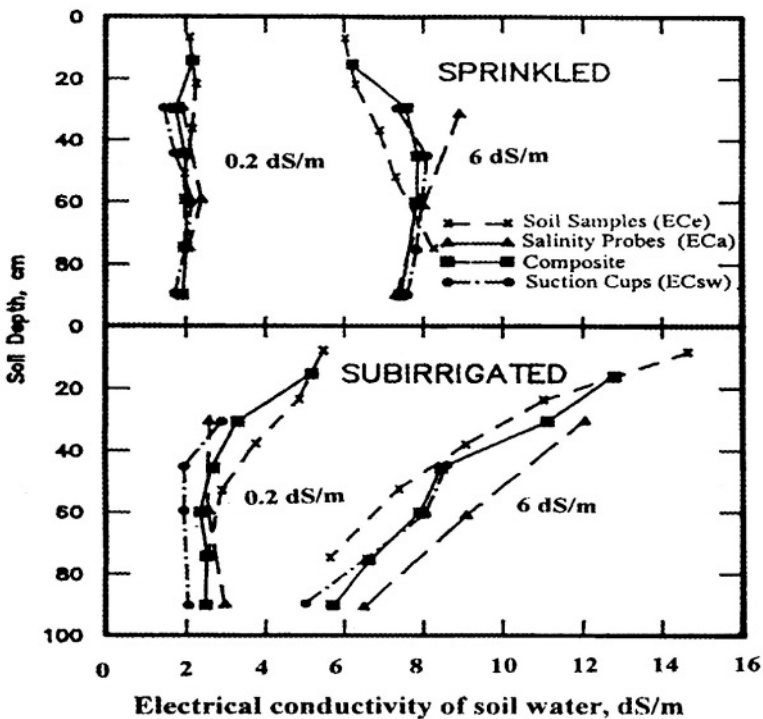


Figure 1. Soil salinity by several EC measurements in maize field plots irrigated by two methods and two water salinities, ECw of 0.2 and 6 dS/m (Hoffman *et al.*, 1983).

suction cup (EC_{sw}), and 4-electrode salinity probe (ECa). The maize plots were irrigated with two waters having an EC (EC_w) of 0.2 and 6 dS/m at a higher than normal LF (Leaching Fraction) of about 0.5 to maintain a comparatively uniform soil salinity in the sprinkled plots to ascertain salt tolerance of corn in peaty soils. A composite salinity is also given that is the mean value of the three methods of measuring soil salinity. The distribution of accumulated salts in the rootzone (in this case, depth to water table) is shown to vary with irrigation method. The average rootzone composite EC in the sprinkled plot was 1.9 dS/m for EC_w of 0.2 dS/m and 7.3 dS/m for EC_w of 6 dS/m while that in the sub-irrigated plots was 3.0 dS/m for EC_w of 0.2 dS/m and 8.6 dS/m for EC_w of 6 dS/m.

Figure 2 (Rhoades, 1972) illustrates the dynamic changes in soil water at the 6 - cm soil depth and soil salinity at the 40 - and 80 - cm depth in the rootzone of alfalfa (*Medicago sativa*) grown over several irrigation intervals. The soil water potential, or tensiometer potential, goes through a cyclical pattern. It rises with time due to rootwater extraction and abruptly decreases with irrigation. A similar cyclical pattern is shown for EC_{sw}.

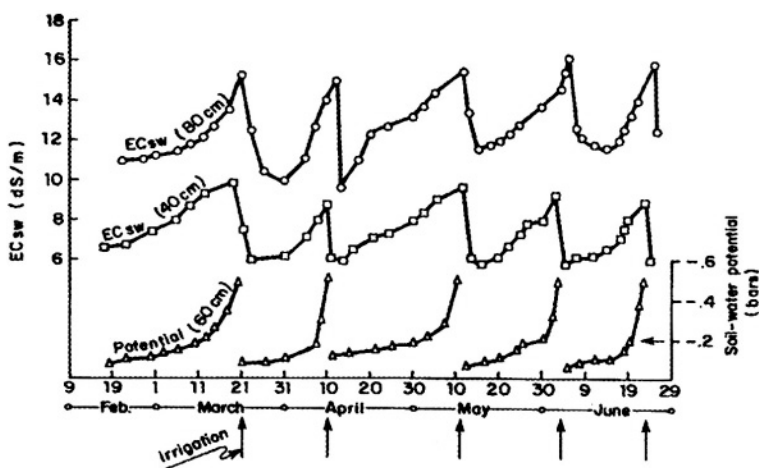


Figure 2. Dynamic changes in soil salinity and soil water potential at one depth in a lysimeter cropped with alfalfa (Rhoades, 1972).

The EC rises due to evapoconcentration from rootwater extraction, and abruptly decreases due to dilution from applied irrigation water. The EC_{sw} is smaller at the 40 cm depth than at 80 cm in this surface irrigated lysimeter because the surface rootzone is a zone of salt leaching while the deeper rootzone is the zone of salt accumulation. This point will be expanded later in Section 2.4.2.

2.2.3 SALINE AND SODIC SOILS

Table 1 gives a classification of salt-affected soils (US Salinity Laboratory, 1954). Saline soils are soils that contain excessive salinity ($E_{ce} > 4$ dS/m) that reduces the availability of soil water due to osmotic effects and affect plant growth and yields. This soil salinity criterion is a relative one because there are substantial differences in salt tolerance among plants. For example, the threshold E_{ce} for bean (*Phaseolus vulgaris*) is 1.0 dS/m, for alfalfa (*Medicago sativa*) 2.0 dS/m and for barley (*Hordeum vulgare*) 8.0 dS/m (Maas, 1990).

The threshold E_{ce} is the soil salinity above that the yield of crops declines. In addition, some of the accumulated constituents such as Na, Cl and B may be toxic to certain crop plants requiring toxic ion threshold levels, too.

TABLE 1. Classification of salt-affected soils and distinguishing properties (US Salinity Laboratory, 1954).

Class	E_{ce} dS/m	ESP %	pHs
Nonsaline	< 4	< 15	< 8.5
Saline	> 4	< 15	< 8.5
Sodic	< 4	> 15	> 8.5
Saline-sodic	> 4	> 15	< 8.5

E_{ce} = EC of extract of saturated soil paste

ESP = (Exch. Na/ CEC)*100 in meq/100 g

pHs = pH of saturated soil paste

Sodic soils are those that contain excessive levels of adsorbed sodium ($ESP > 15\%$) that cause unfavorable soil physical conditions such as decreased water intake rates and may be toxic to plants. ESP is defined as (Exchangeable Na/CEC)*100 in units of meq/100g soil. CEC is the cation exchange capacity of the soil. Likewise, the criterion of ESP (exchangeable sodium percentage) is a relative one because physical effects from Na vary among types of soil minerals. For example, smectite (2:1 layer silicate) containing soils tend to disperse more readily from Na than kaolinite (1:1 layer silicate) containing soils. Some salt-affected soils are both saline and sodic while others are saline-nonsodic or nonsaline-sodic. Sodic soils have a pH greater than 8.5 because of the presence of either Na_2CO_3 or NaOH. Table 1 should be used as a general guide.

The FAO soil classification system utilizes solonchaks for soils with excess soluble salt accumulation and solonetz for soils with excess sodium. The US soil taxonomy classifies salt-affected soils into the soil order aridisols while the Canadian system uses solonetzic.

2.2.4 NATURAL SOURCES OF SALTS AND PRIMARY SALINIZATION

The origin of soil salts may be natural or anthropogenic (human-induced). The former results in primary salinization and the latter, secondary salinization. The presence of salts in soils is due to a complex interaction of geochemistry, hydrology, and vegetation. The principle natural source of salinity is the geochemical weathering of minerals present in rocks, sediments and soils, producing dissolved mineral salts. The principle

salt sink for dissolved mineral salts is the world oceans. Inland salt sinks include saline soils, saline ground water bodies, saline lakes, saline marshlands, saline playas, and salt domes.

As pointed out earlier, salinity present in soils is subjected on the one hand to evaporative concentration from surface evaporation and evapotranspiration and on the other hand to dilution from rainfall and snowmelt (atmospheric precipitation). Since soils in arid regions are subjected to high evaporation/precipitation ratios, they tend to be saline while soils in humid regions are subjected to low evaporation/precipitation ratios and they tend to be non-saline.

The source of salts in many arid zone sedimentary rocks is marine in origin. The landscape in geologic times was inundated by the ancient seas and was uplifted due to tectonic forces and these rocks contain residual sea salts and minerals. When seawater is desiccated, one of the first minerals to precipitate out in copious quantities is calcite (CaCO_3) because of its low solubility (Smith *et al.*, 1994). Another sparingly soluble mineral to precipitate out in the initial desiccation stages is gypsum ($\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$). With further drying, minerals of higher solubility are deposited, including halite (NaCl), thenardite (Na_2SO_4), and bloedite ($\text{Na}_2\text{Mg}(\text{SO}_4)_2 \cdot 4\text{H}_2\text{O}$), depending on initial chemical composition of the water. Thus, saline soils are typically dominated by Na^+ , Mg^{2+} , Cl^- , and SO_4^{2-} and not by Ca^{2+} , HCO_3^- , and CO_3^{2-} ions.

The genesis of sodic soils is more complex. Some sodic soils may have formed from the leaching of saline soils rich in Na, resulting in excess ESP. Others may have been formed from the chemical weathering of igneous rocks producing NaHCO_3 under non-saline conditions and forming Na_2CO_3 upon evapoconcentration. Another pathway involves the biological formation of Na_2CO_3 by the reduction of SO_4^{2-} to S^{2-} (Whittig and Janitsky, 1963).

Other natural sources of soil salts may include atmospheric deposition of oceanic salts along coastal areas, seawater intrusion into estuaries and streams due to tidal events, seawater intrusion into ground water basins in coastal areas due to excessive ground water pumping, rising ground waters in low-lying topography from saline aquifers, salt domes and saline lakes and playas, and rainfall leaching of saline land forms.

2.2.5 ANTHROPOGENIC SOURCES OF SALTS AND SECONDARY SALINIZATION

Anthropogenic sources of soil salts include salts present in irrigation waters, and residual salts from soil and water amendments (gypsum, elemental sulfur, acids), animal wastes (manures and wash waters), chemical fertilizers, and applied sewage sludges and effluents. Another source of salts is disposal of oil and gas field brines.

Secondary salinization of soils may arise from a number of human-induced practices and management in regard to irrigation and drainage, grazing, and deforestation. In the planning and operation of most large-scale irrigation projects, a drainage system is not provided until problems of water logging occur. The typical scenario includes diversion of irrigation water from surface water supplies and deep percolation of irrigation water, oftentimes from over-irrigation, resulting in the filling of the vadose zone (unsaturated underground basin). After decades of irrigation, the vadose zone is filled to its capacity

and shallow ground water encroaches into the crop root zone, developing waterlogged conditions. Salinity is frequently associated with water logging (Tanji, 1990 b). Under high water table conditions, there is upward movement of salts at least some of the time due to evaporation and rootwater extraction. In irrigated river basins, the deep percolation drainage water in up-slope areas typically moves vertically downwards and then laterally to down-slope areas due to restricting layers in the soil or substrata. Thus, water logging and secondary salinization in down-slope areas may be partly contributed by up-slope irrigators who do not typically have a drainage problem (San Joaquin Valley Drainage Program, 1990).

A second condition is hydrologic imbalance from disturbing the natural vegetation. Pasture lands that have been overgrazed usually result in an imbalance between precipitation and evapotranspiration. If the overgrazed land overlies shallow ground water, the ground water may rise upwards and the dissolved mineral salts will tend to accumulate in the surface soils forming saline or sodic or saline-sodic soils. In other situations when crop plants that have smaller ET replace the natural vegetation (e.g., forest), seepage to the ground water basin is enhanced. The resultant seepage reappears down slope, frequently carrying salines as in dryland saline seeps in western USA (Halvorson, 1990) and increase in stream salinity as in Western Australia (Holmes and Talsma, 1981). The salts in the western USA is derived from chemical weathering of soils while that in Western Australia is deposition of sea salts from precipitation originating from the Indian Ocean.

2.3 Chemistry of Saline Soils

2.3.1 ION PAIRS

Figure 3 describes the complex chemical interactions that can take place between the soil solution, primary soil mineral, exchange complex (charged secondary clay minerals and organic matter), and gaseous phases. In the soil solution, dissolved mineral salts exist as completely dissociated ions such as Na^+ and Cl^- , partly dissociated ion pairs such as NaSO_4^- and CaHCO_3^+ , and neutrally charged ion pairs such as CaCO_3^0 and CaSO_4^0 . These interactions occur rapidly enough that we can neglect kinetics and assume chemical equilibrium conditions to prevail in soil chemistry.

Table 2 gives K_d , the dissociation constant, and pK_d values for ion pairs. Lower case p stands for negative log as in pH, pK_{sp} , and pE .

A typical dissociation reaction and its equilibrium constant is



$$K_d = \frac{a_{\text{Ca}} a_{\text{SO}_4}}{a_{\text{CaSO}_4^0}} = \frac{m_{\text{Ca}} \gamma_{\text{Ca}} m_{\text{SO}_4} \gamma_{\text{SO}_4}}{m_{\text{CaSO}_4^0} \gamma_{\text{CaSO}_4^0}} \cong \frac{m_{\text{Ca}} \gamma_{\text{Ca}} m_{\text{SO}_4} \gamma_{\text{SO}_4}}{m_{\text{CaSO}_4^0}} \quad (2)$$

where the neutrally charged species dissociates completely into free ions. A new equilibrium concentration of CaSO_4° is formed if the concentration of Ca^{2+} , SO_4^{2-} or both changes. The K_d is defined as the activity concentration of free Ca^{2+} and SO_4^{2-} ions divided by the activity concentration of the ion pair CaSO_4° . Activity of the solute species i (a_i), in turn, is defined by the product of its analytical concentration (m_i) in M/L and ion activity coefficient (γ_i) in L/M so that a_i is dimensionless. Since γ_i of neutrally charged ion pairs is taken at unity, $\gamma_{\text{CaSO}_4^\circ}$ drops out and the K_d reduces to the far right hand side in equation (2).

The single-ion activity coefficient γ_i in heterogeneous electrolytes up to about 0.1M ionic strength may be obtained from the Davies equation,

$$\log \gamma_i = -0.509 z_i^2 \left[\frac{-0.509 z_i^2 I^{0.5}}{1 + I^{0.5}} - 0.2 I \right] \quad (3)$$

where z_i is the charge of the ion and I , the ionic strength in M/L, is obtained from

$$I = 0.5 \sum m_i z_i^2 \quad (4)$$

K_d gives the strength of ion pairing, but it may be more clearly viewed by K_a , association constant, which is the reciprocal of K_d . The literature typically contains K_d or pK_d and not K_a . Ion pairing can not be determined directly (Davies, 1962) and are indirectly inferred from other measures such as specific conductance of electrolytes

TABLE 2. The dissociation constant (K_d) of ion pairs at 25°C (Smith, 1989).

Species	Dissociation constant K_d	pK_d	Association constant K_a
CaSO_4°	4.90×10^{-3}	2.31	204
MgSO_4°	5.89×10^{-3}	2.23	170
NaSO_4^-	2.00×10^{-1}	0.7	5.00
KSO_4^-	1.41×10^{-1}	0.85	7.09
CaCO_3°	7.08×10^{-4}	3.15	1,410
MgCO_3°	1.17×10^{-3}	2.93	855
NaCO_3^-	5.37×10^{-2}	1.27**	18.6
$\text{Na}_2\text{CO}_3^\circ$?	?	?
KCO_3°	1.047	-0.02	0.955
CaHCO_3^+	5.62×10^{-2}	1.25	17.8
MgHCO_3^+	6.92×10^{-2}	1.16	14.5
NaHCO_3°	1.738	-0.24	0.575
KHCO_3°	?	?	?
CaCl^+	10	-1.0	0.100
CaCl_2°	0	0	0
MgCl^+	3.02×10^{-1}	0.52*	3.12
MgCl_2°	1.072	-0.03	0.933
NaCl°	7.59×10^{-4}	3.12*	1,318
KCl°	5.012	-0.70	0.199

* from Pytkowic (1983)

** from Nordstrom *et al.* (1990)

(Tanji, 1969 a) and mineral solubility (Tanji, 1969 b). The extent of ion pairing in a given solution is regulated by the concentration levels of cations and anions present and they, in turn, are regulated by the solubility of the mineral solid phase. Therefore, the solution chemistry of associated electrolytes is complex in contrast to non-associated electrolytes in which solute species exist as only free ions.

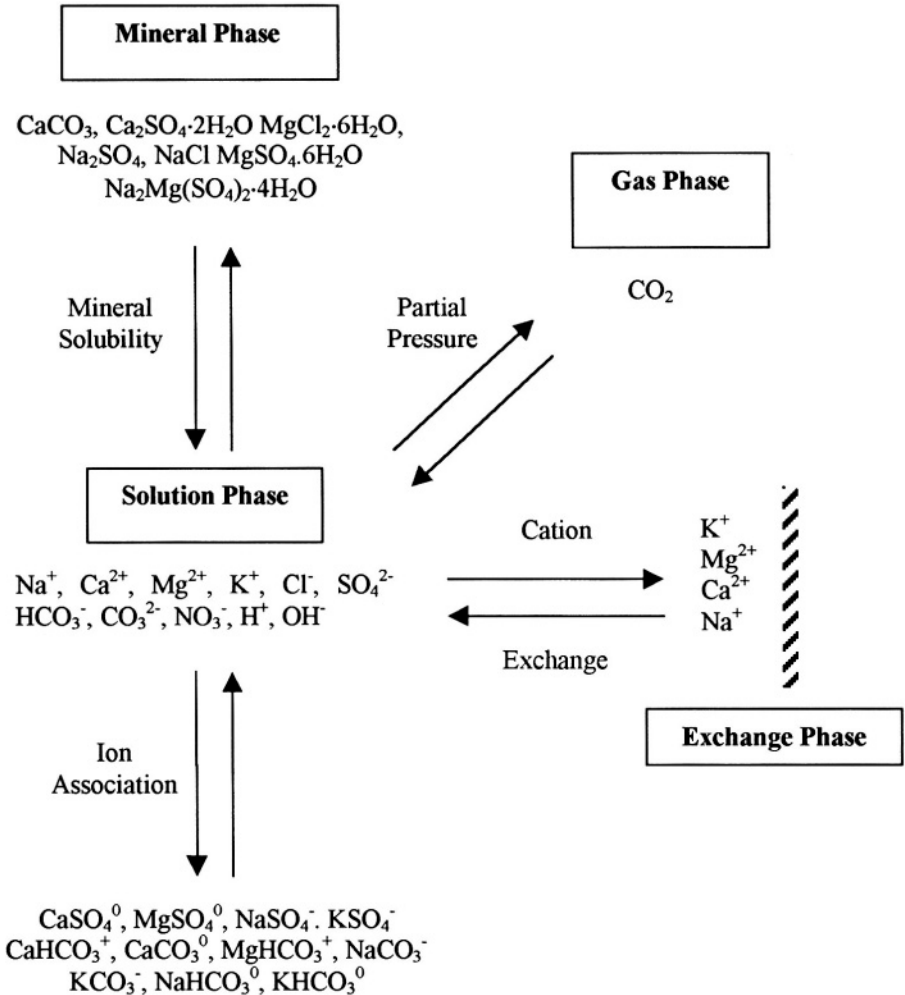


Figure 3. Interactive chemical reactions between solution, mineral, exchanger, and gas phases in saline soils (Tanji, 1990 b).

2.3.2 MINERAL SOLUBILITY

Table 3 contains evaporite (sometimes called evaporate) minerals that form from major solutes in a desiccating environment. The solubility product constant, K_{sp} , is the ion activity product of the cations and anions in a saturated solution of the mineral in question, extrapolated to zero ionic strength. In general, chloride and sulfate minerals (except, gypsum and anhydrite) are highly soluble and carbonate minerals, less soluble. At lower salinities, the predominant minerals deposited upon evapoconcentration of soil solutions are calcite, or aragonite if the Ca/Mg ratio is less than about unity, and gypsum. At elevated salinities, e.g. $EC > 150$ dS/m, thenardite, glauberite, bloedite and halite are common minerals formed. Most saline agricultural soils have EC_e less than about 20 dS/m in the rootzone, but a thin layer of surface saline soils may have elevated EC_e forming salt efflorescence (salt crust) when the moisture gradient is upwards as in furrow-irrigated beds and subjected to high evaporation rates.

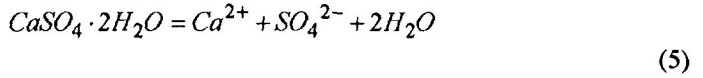
The total solubility of minerals is affected not only by its K_{sp} but also by ionic strength, ion pairs, and common ions. For example, Tanji (1969 b) has extensively studied the solubility of gypsum ($CaSO_4 \cdot 2H_2O$) in distilled water and in the presence of external salt solutions. Figure 4 shows that the total solubility of gypsum in distilled water is 15.34 mM/L and increases in the presence of increasing external chloride salt solutions.

TABLE 3. The solubility product constant of evaporite minerals in saline environments (Smith, 1989).

Mineral species	Chemical formula	K_{sp}	pK (-log K_{sp})
Halite	NaCl	3.72×10^1	-1.57
	$CaCl_2 \cdot 6H_2O$?	?
Bischofite	$MgCl_2 \cdot 6H_2O$	2.85×10^4	-4.455
Carnalite	$KMgCl_3 \cdot 6H_2O$	2.14×10^4	-4.330
Sylvite	KCl	7.943	-0.9
Thenardite	Na_2SO_4	5.15×10^{-1}	0.288
Mirabilite	$Na_2SO_4 \cdot 10H_2O$	5.96×10^{-2}	1.225
Gypsum	$CaSO_4 \cdot 2H_2O$	2.62×10^{-5}	4.581
Anhydrite	$CaSO_4$	4.34×10^{-5}	4.362
Kieserite	$MgSO_4 \cdot 4H_2O$	7.53×10^{-1}	0.123
Hexahydrate	$MgSO_4 \cdot 6H_2O$	2.32×10^{-2}	1.635
Epsomite	$MgSO_4 \cdot 7H_2O$	1.32×10^{-2}	1.881
Arcanite	K_2SO_4	1.67×10^{-2}	1.776
Bloedite	$NaMg(SO_4)_2 \cdot 4H_2O$	4.50×10^{-3}	2.347
Glauberite	$Na_2Ca(SO_4)_2$	5.69×10^{-6}	5.245
Polyhalite	$K_2MgCa_2(SO_4)_4 \cdot 2H_2O$	1.80×10^{-14}	13.744
Calcite	β - $CaCO_3$	3.93×10^{-9}	8.406
Aragonite	λ - $CaCO_3$	6.04×10^{-9}	8.219
Magnesite	$MgCO_3$	1.50×10^{-8}	7.825
Nesquehonite	$MgCO_3 \cdot 3H_2O$	6.81×10^{-6}	5.167
Nahcolite	$NaHCO_3$	3.95×10^{-1}	0.403
Kalicinite	$KHCO_3$	1.91×10^1	-1.281
Burkeite	$Na_2CO_3(SO_4)_2$	1.91	-0.281
Natron	$Na_2CO_3 \cdot 10H_2O$	1.50×10^{-1}	0.825
Trona	$Na_2(CO_3) \cdot NaHCO_3 \cdot 2H_2O$	4.43×10^{-12}	11.354
Potassium Trona	$K_2Na(HCO_3)_2 \cdot 2H_2O$	7.89×10^{-10}	9.103

In contrast, the total solubility of gypsum decreases in the presence of increasing external salt solutions containing an ion common with the dissolution products of gypsum, Ca^{2+} or SO_4^{2-} .

Table 4 contains data to illustrate this complex solubility behavior of gypsum. The computations were carried out with a solution chemistry model utilizing equations given above. The solubility of gypsum, equation (5), based on its K_{sp} of 2.4×10^{-5} at 25°C (2.62×10^{-5} in Table 2; the values of K_d and K_{sp} may differ somewhat among literature references) gives 10.44 mM/L Ca^{2+} and SO_4^{2-} as its dissolution products. However, the measured total solubility of gypsum in distilled water is 15.3 mM/L; calculated is 15.34 mM/L. The difference between 15.34 and 10.44 mM/L is due to the formation of ion pair from the dissolution product, equation (1), driving the reaction in equation (5) to the right hand side resulting in more gypsum going into solution.



The equilibrium constant expression for equation (5) is the K_{sp} , equation (6).

$$K_{sp} = \frac{a_{\text{Ca}} a_{\text{SO}_4} a_{\text{H}_2\text{O}}^2}{a_{\text{CaSO}_4 \cdot 2\text{H}_2\text{O}}} = a_{\text{Ca}} a_{\text{SO}_4} \quad (6)$$

Since the activity of H_2O and pure solid phase are taken as unity by standard state conventions, the K_{sp} of gypsum reduces to the far right-hand side of equation (6).

Table 4 gives calculated values for various solution parameters. In a solution saturated with respect to sparingly soluble gypsum, the maximum concentration of CaSO_4° that can form is 4.898 mM/L based on equations (2) and (6):

$$K_d = \frac{a_{\text{Ca}} a_{\text{SO}_4}}{m_{\text{CaSO}_4}^\circ} = \frac{K_{sp}}{m_{\text{CaSO}_4}^\circ} \quad (7)$$

where the numerator is the definition of K_{sp} of gypsum (equation (6)). Inserting numerical values of K_{sp} (2.4×10^{-5}) and K_d (4.9×10^{-3}) in equation (7) results in $m_{\text{CaSO}_4}^\circ$ of $4.898 \times 10^{-3} \text{M}$. Using equation (3), γ_i for divalent ions (γ_2) is 0.469. The activity concentration of Ca^{2+} and SO_4^{2-} (4.896×10^{-3}) is obtained from the product of 10.44 mM/L and 0.469 L/M. The product of a_{Ca} and a_{SO_4} is 2.4×10^{-5} , the K_{sp} of gypsum. The net increase in solubility of gypsum above that given by K_{sp} is the ion pair. Therefore, the calculated total solubility of gypsum in distilled water is 15.34 example, the K_a of MgSO_4° (170) is nearly comparable to CaSO_4° (204) but the maximum concentration possible of MgSO_4° is about three orders of magnitude larger

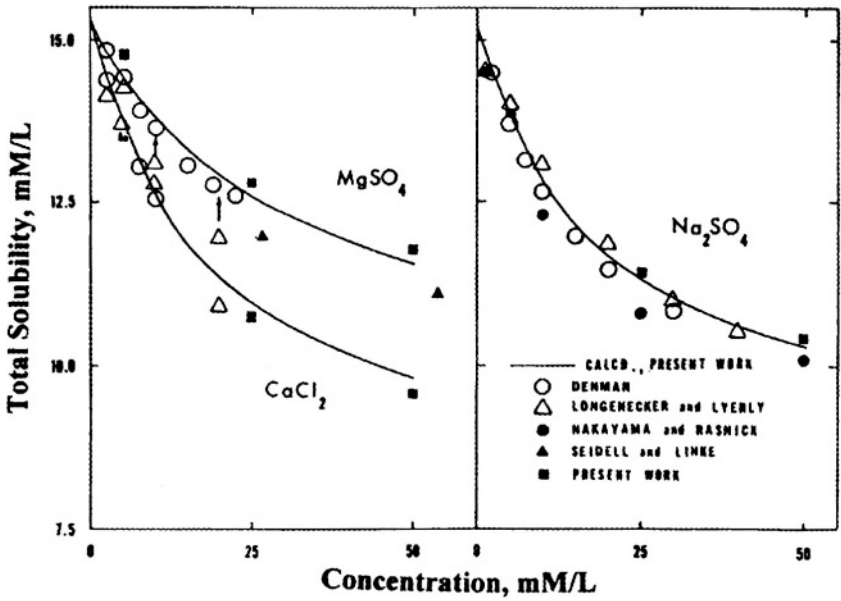
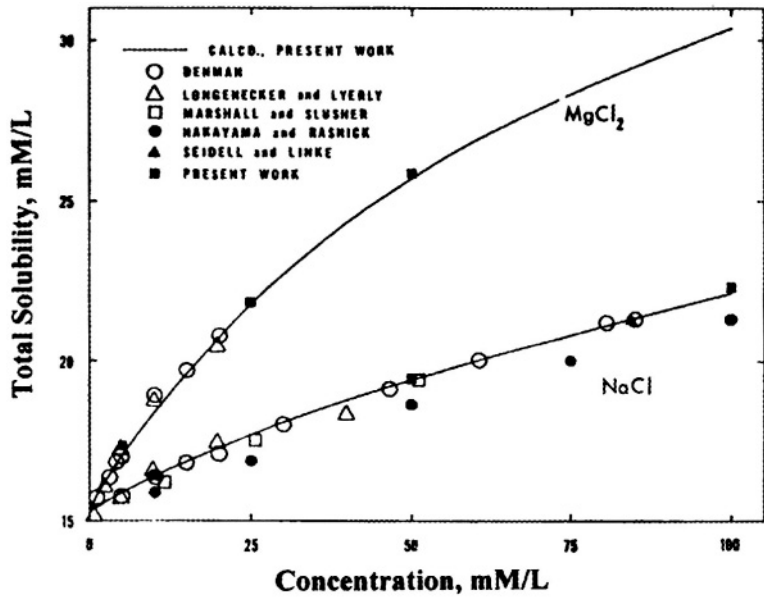


Figure 4. The solubility of gypsum in the presence of external chloride salt solutions and common-ion containing salt solutions (TANJI, 1969 b)

mM/L comprised of 10.44 mM/L free ion (68%) and 4.90 mM/L ion pair (32%). It should be pointed out that Ksp of other sulfate minerals are much higher than gypsum and thus the maximum concentration of other sulfate ion pairing is greater. For example, the Ka of MgSO_4° (170) is nearly comparable to CaSO_4° (204) but the maximum concentration possible of MgSO_4° is about three orders of magnitude larger than CaSO_4° in saturated solutions of hexahydrate because its Ksp is 2.32×10^{-2} (Table 3). The Ka of NaSO_4^- is smaller (5.00) and thus in saturated solutions of thenardite the maximum concentration of NaSO_4^- possible is about 22 times greater than CaSO_4° because its Ksp is 5.15×10^{-1} (Table 3).

TABLE 4. Solubility of gypsum in single salt solutions (Tanji, 1969 b).

Parameter	Gyp. + Distilled water	Gyp. + 0.05 M NaCl	Gyp. + 0.05 M MgCl_2	Gyp. + 0.05 M MgSO_4
Calcd. Ca^{2+} , mM/L	10.44	14.52	16.85	7.68
Calcd. SO_4^{2-} , mM/L	10.44	13.57	13.26	23.97
Calcd. CaSO_4° , mM/L	4.898	4.898	4.898	4.898
Calcd. NaSO_4^- , mM/L		0.95		
Calcd. MgSO_4° , mM/L			3.59	8.71
Calcd. Davies γ_2 , L/M	0.469	0.349	0.328	0.361
Calcd. a_{SO_4} , 10^{-3}	4.896	4.74	4.35	8.65
Calcd. a_{Ca} , 10^{-3}	4.896	5.07	5.52	2.77
Measd. a_{Ca} , 10^{-3}	4.8	5.0	5.8	2.9
Calcd. Total Soly., mM/L	15.34	19.42	21.75	12.58
Measd Total Soly., mM/L	15.30	19.42	21.82	12.78
Calcd. Total Soly., mM/L	15.34	19.42	21.75	12.58
Soly. from Ksp, mM/L	10.44 (68%)	10.44 (54%)	10.44 (48%)	10.44 (83%)
Net increase, mM/L	4.90	8.98	11.31	
Total ion pairs, mM/L	4.90 (32%)	5.85 (30%)	8.49 (39%)	
Soly. due to I-effect, mM/L		3.13 (16%)	2.82 (13%)	

Based on Ksp of gypsum = 2.4×10^{-5} , Kd of CaSO_4° = 4.9×10^{-3} , Kd of NaSO_4^- = 1.9×10^{-1} , Kd of MgSO_4° = 6.1×10^{-3} , and the Davies equation for γ_i .

In the presence of external chloride salt solutions, the solubility of gypsum is greater than in distilled water. For illustrative purposes, Table 4 gives calculated and measured data for the system gypsum + 0.05 M NaCl and gypsum + 0.05 M MgCl_2 . In the NaCl system, the total solubility of gypsum, 19.42 mM/L, is made up of 14.52 mM/L Ca^{2+} and 4.90 mM/L CaSO_4° or 13.57 mM/L SO_4^{2-} , 4.90 mM/L CaSO_4° and 0.95 mM/L NaSO_4^- . The total solubility of gypsum in 0.05M NaCl is attributed to 54% from Ksp of gypsum, 30% from ion pairs and 16% from ionic strength effect. In contrast, the total solubility of gypsum in the presence of 0.05 M MgCl_2 is greater than in 0.05 M NaCl, 21.75 vs. 19.42 mM/L. In the MgCl_2 system, the total solubility of gypsum is comprised of 48% from Ksp, 39% from ion pairs and 13% from I-effect. The difference in solubility of gypsum in these chloride salt solutions is attributed to differences in ion pair formation, $\text{MgSO}_4^\circ > \text{NaSO}_4^-$, and ionic strength, $I_{\text{MgCl}_2} > I_{\text{NaCl}}$, i.e., the ionic strength of 2-1 electrolyte (MgCl_2) is greater than 1-1 electrolyte (NaCl).

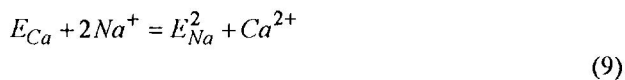
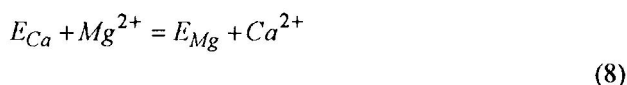
In the presence of external MgSO_4 salt solution, the solubility of gypsum is repressed due to the common ion effect. Adding SO_4^{2-} to the right hand side of equation (5) will drive the reaction to the left and the solubility of gypsum will be depressed. Alternatively, the addition of MgSO_4 increases the a_{SO_4} in the far right hand side of equation (6) so the a_{Ca} must necessarily decrease because K_{sp} is a fixed value at a given temperature. When the a_{Ca} is decreased, the solubility of gypsum is repressed. For the system gypsum + 0.05M MgSO_4 , the calculated a_{SO_4} is 8.65×10^{-3} and a_{Ca} is 2.77×10^{-3} , the product being 2.4×10^{-5} , the K_{sp} of gypsum. Although there was a net increase of 2.14 mM/L over that from K_{sp} and a total of 13.61 mM/L of ion pairs, the effects of ion pair and ionic strength on total solubility are overridden by the common ion effect.

The data presented in Table 4 indicates chemical speciation is important when considering the total solubility of minerals. Moreover, it is likely that plant uptake of free ions vs. partly charged ion pairs vs. neutrally charged ion pairs may differ.

2.3.3 CATION EXCHANGE

Arid zone soils typically have a net negative charge and can attract positively charged ions from the soil solution. The charges emanate from hydroxyl and other functional groups on colloidal surfaces that are largely pH dependent as well as isomorphous substitution of cations in the clay crystal structure by another cation of lesser charge that are largely permanent charges. The pH dependent charges are found in humus, 1:1 layer silicates like kaolinite, and oxides of iron, manganese and aluminum. In contrast, the permanent charges are found in 2:1 layer silicates such as smectites. The cation exchange capacity (CEC) of arid zone soils may range from less than 4 meq/100g in coarse textured sandy soils to over 40 meq / 100 g in finer textured clayey soils. Soils rich in organic matter may have elevated CEC, too.

Cation exchange occurs when a given cation on the exchanger phase is replaced by a different cation from the solution phase. Cation exchange occurs when there is a shift in cationic composition in the solution phase. Divalent cations are held more strongly on the exchanger phase than monovalent cations. Equations (8) and (9), respectively, show cation exchange between Exchangeable Ca (E_{Ca}) and solution Mg^{2+} and E_{Ca} and solution Na^+ .



The literature contains a number of differing approaches to describing cation exchange (Sposito, 1981). The equilibrium constant for these exchange equations gives the extent of exchange that can take place. The term selectivity constant is oftentimes used in place of equilibrium constant in the exchange equations because it may not be constant over a wide range of concentration. For illustrative purposes, a thermodynamic type of cation exchange equation (Krishnamoorthy *et al.*, 1949) is given herein:

$$K_{Ca-Mg} = \frac{a_{Ca}E_{Mg}}{a_{Mg}E_{Ca}} \quad (10)$$

$$K_{Ca-Na} = \frac{a_{Ca}E_{Na}^2}{a_{Na}^2E_{Ca}[E_{Na} + 1.5E_{Ca}]} \quad (11)$$

Paul *et al.* (1967) determined cation exchange constant in five California soils of different CEC and soil mineralogy. The CEC ranged from 3.0 (Oakley sand) to 39.1 meq/100g (Sacramento clay). The K_{Ca-Mg} ranged from 0.46 to 0.67, meaning only slight preference on the exchanger for either Ca or Mg. K_{Ca-Na} ranged from 0.14 to 0.18, meaning strong preference for Ca over that of Na. The exchange between E_{Mg} and Na^+ is obtained from the ratio of K_{Ca-Na} to K_{Ca-Mg} .

2.3.4 SOIL SALINITY AS AFFECTED BY CHANGE IN SOIL MOISTURE CONTENT

Mention was made earlier that salinity typically does not change in direct proportion to change in soil water contents. Tanji *et al.* (1967) developed a model to predict salt concentrations at varying soil moisture contents by considering solution chemistry, sulfate ion pairing, solubility of gypsum and cation exchange. Paul *et al.* (1966) validated this model.

Table 5 contains illustrative calculated and measured data for Hanford sandy loam and Yolo clay loam. The CEC for the Hanford soil is 4.21 meq/100 g and for Yolo, 18.42 meq/100 g. The soils were salinized by adding NaCl (10% of CEC) and gypsum (25% of CEC). Extracts of soil: water suspensions at 1:1 ratio by weight (1:1) and at saturation percentage (SP) were analyzed. The input data for the model is the measured chemistry from 1:1 soil-water content and the predicted salinity is at SP that is compared to measured values at SP for model validation. The concentration of all the soluble and exchangeable species and gypsum are expected to be altered with a reduction in soil moisture, simulating evapoconcentration. The evapoconcentration factor for the Hanford soil is 5.55 (1:1/SP or 101.0/18.2) and Yolo soil 3.11 (105.9/34.1). If TSC changes in direct proportion to change in soil water content, the TSC for Hanford soil would be 82.4 meq/L (14.85 meq/L x 5.55) at SP, but the measured TSC is 41.1, and TSC for Yolo soil would be 216 meq/L (69.4 meq/L x 3.11), but the measured TSC is 67.7 meq/L. Clearly, the dissolved mineral salts are reactive in the soil solution.

The exchangeable cation concentration did not remain constant when the moisture content was decreased because gypsum which was initially absent in Hanford soil precipitated out (0.61 meq/100 g), while gypsum concentration in Yolo soil increased from 0.28 to 3.59 meq/100 g. Thus, the soluble Ca^{2+} and SO_4^{2-} ions were decreased by the amount of gypsum precipitation. A change in cation concentration in the solution phase will result in shifts in the exchangeable cations. The only constituent assumed to

be non-reactive is Cl^- , but there is some discrepancy between measured and calculated values (28.6 vs. 24.4 meq/L for Hanford soil and 67.7 vs. 57.4 meq/L for Yolo soil). This discrepancy may be attributed to analytical error for Cl^- but it is too large and most probably is due to lack of taking representative samples of the soil in preparing the two extracts at 1:1 and SP.

TABLE 5. Measured and predicted changes in soluble and exchangeable ions and gypsum content with change in soil water content (Paul *et al.*, 1966).

Soil type	Hanford sandy loam			Yolo clay loam		
CEC, meq/100g		4.21			18.42	
$\text{K}_{\text{Ca-Mg}}$		0.54			0.67	
$\text{K}_{\text{Ca-Na}}$		0.143			0.148	
	Measd.	Measd.	Pred.	Measd.	Measd.	Pred.
Water content, %	101.0	18.2	18.2	105.9	34.1	34.1
Soluble ions, meq/L						
Ca	10.5	34.5	35.4	30.2	40.2	40.7
Mg	3.02	12.1	9.74	26.4	49.4	43.8
Na	4.35	20.8	16.6	12.8	33.5	28.3
TSC	14.85	67.4	61.74	69.4	123.1	112.8
Cl	4.40	28.6	24.4	18.5	67.7	57.4
SO_4	14.2	41.1	41.6	51.1	52.7	56.0
SAR, meq/L ^{0.5}	1.7	4.3	3.5	2.4	5.0	4.5
Exchangeable ions, meq/100g						
Ca	3.62	3.42	3.35	10.66	9.18	8.96
Mg	0.56	0.64	0.69	7.20	8.32	8.50
Na	0.03	0.09	0.17	0.56	0.97	0.95
ESP, %	0.7	2.1	4.0	3.0	5.3	5.2
Gypsum, meq/100g	0.00	0.61	0.68	0.28	3.59	3.79

Soils artificially salinized with NaCl (10% of CEC) and gypsum (25% of CEC). Measured values obtained in extracts of 1:1 soil water suspension (near 100% water content) and saturated soil paste (18.2 or 34.1 % moisture content). Predicted values based on soil chemistry computer model (TANJI *et al.*, 1967).

SAR (Sodium Adsorption Ratio) given below, in units of either $(\text{mM/L})^{0.5}$ or $(\text{meq/L})^{0.5}$, is a soil solution or water sodicity parameter while ESP is a soil sodicity parameter. These two sodicity parameters are related because of cation exchange. SAR is more commonly measured than ESP because the latter is more time consuming to determine analytically. Empirical and nomograph relations are found in the Salinity Handbook (US Salinity Laboratory, 1954). SAR is defined as

$$\text{SAR} = \frac{\text{Na}^+}{[\text{Ca}^{2+} + \text{Mg}^{2+}]^{0.5}} \text{ in units of mM/L} \tag{12}$$

$$\text{or SAR} = \frac{\text{Na}^+}{[(\text{Ca}^{2+} + \text{Mg}^{2+})/2]^{0.5}} \text{ in units of meq/L} \tag{13}$$

2.3.5 GEOCHEMICAL AND SOIL CHEMISTRY COMPUTER MODELS

Comprehensive chemical speciation in waters and soils are obtained from computer models such as MINTEQA2 for lower salinity and PHRQPITZ for hypersaline systems. The former utilizes either the Debye-Huckel or Davis equation to calculate ion activity coefficients and the latter, Pitzer equations for hypersaline waters. The models provide detailed description on speciation (free ions and ion pairs or complexes) and the degree to which the water is saturated with respect to minerals (IAP/Ksp). These models are reviewed in Jenne (1979), Melchior and Bassett (1990), and Loeppert *et al.* (1995). Plant scientists may be interested in using MINTEQA2 for chemical speciation.

2.4 Soil Salinity in Irrigated Soils

2.4.1 SALT DISTRIBUTION PATTERNS IN SOILS FROM DIFFERING IRRIGATION APPLICATION METHOD

Figure 5 (Ayers and Westcot, 1985) shows a schematic drawing of salt accumulation patterns in near surface and deeper rootzone of soils irrigated by differing methods of application of irrigation water. The unique salt distributions reflect dominant water flow patterns and surface evaporation and evapotranspiration. Salt accumulation in the near surface soils, as in furrow beds, are typically reduced by sprinkler application. The next section gives further details on rootzone salt distribution.

2.4.2 PROFILE SALINITY AS AFFECTED BY LF AND ROOTWATER EXTRACTION

LF is a commonly used parameter to evaluate salt balance in the crop root zone in those irrigated lands having little or no rainfall. Figure 6 depicts the crop root zone that varies in depth with plant species and soil conditions. Here, the rootzone is considered as a slab. D_{ci} is the depth of crop irrigation, most of which typically infiltrates into the rootzone, with some surface runoffs, D_{sro} . The water that enters the crop rootzone is known as effective crop irrigation, D_{eci} . Of the effective crop irrigation, a large portion is consumed by the crop plants and evapotranspired, D_{cet} or depth of crop ET, and the remaining water will become irrigation drainage, D_{id} , passing out of the rootzone. This is a rather simplified look at field water cycle. It is assumed that the soil has no internal drainage problems and that drainage takes place freely. The entire crop irrigation season will be considered herein.

The seasonal leaching fraction on a water depth basis is defined as

$$LF = \frac{D_{eci} - D_{cet}}{D_{eci}} = \frac{D_{id}}{D_{eci}} \quad (12)$$

For instance, if the seasonal D_{eci} is 1,000 mm (ha.mm/ha) and D_{cet} is 700 mm, D_{id} produced would be 300 mm and the LF would be 0.3, or 30% of the effective crop irrigation.

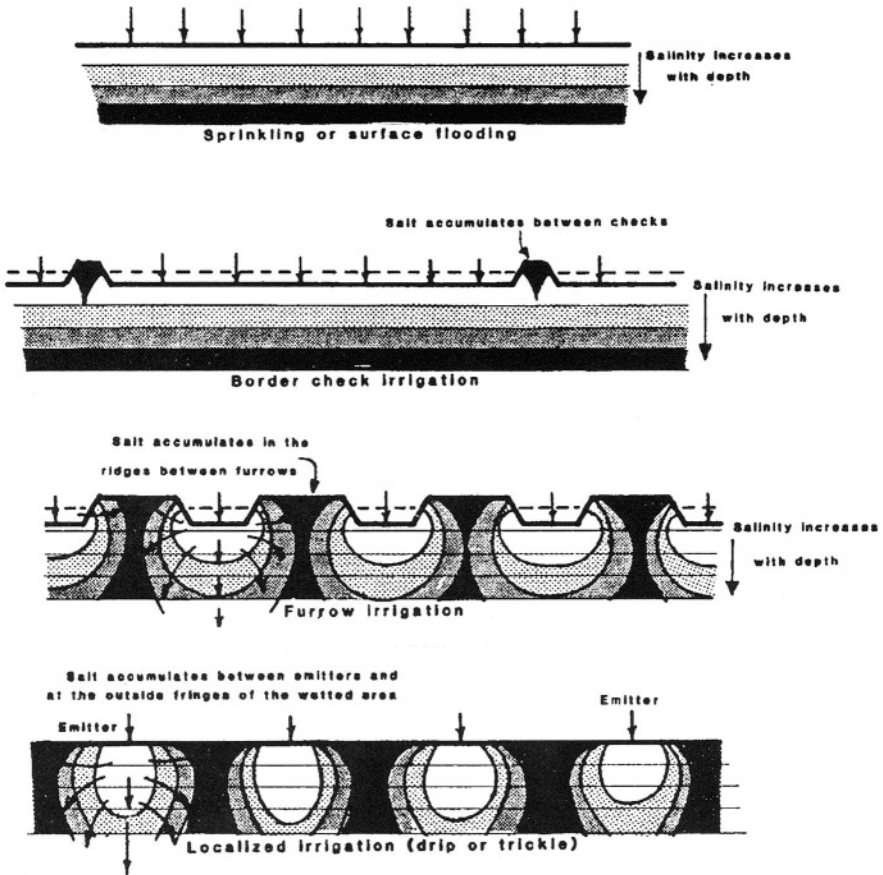


Figure 5. Typical salt accumulation patterns from differing irrigation application method (Ayers and Westcot, 1985).

The seasonal LF on a salinity basis is given as

$$LF = \frac{EC_{iw}}{EC_{id}} \quad \text{or} \quad EC_{id} = \frac{EC_{iw}}{LF} \quad (13)$$

where EC_{iw} is the EC of the applied water and EC_{id} is the EC of the drainage water in dS/m. If EC_{iw} is 1.0 dS/m and LF is 0.3, then, the EC_{id} will be 3.33 dS/m, since the applied water was evapoconcentrated 3.33-fold by Dcet. We will assume that the salinity in the whole rootzone will be the same as irrigation drainage, and that the salts act conservatively and does not participate in mineral precipitation or mineral dissolution. Later, the rootzone will be considered as multi-layered.

TABLE 6. The inverse relations between LF on a water depth basis and LF on a salinity basis.

Deci	Dcet	Did	$LF = Did/Deci$	ECiw	$ECid = ECiw/LF$
mm	mm	mm		dS/m	dS/m
1000	700	300	0.3	1.0	3.33
1000	800	200	0.2	1.0	5.00
1000	900	100	0.1	1.0	10.0

Note that an inverse relation exists between LF on a salinity basis and LF on a water depth basis. An increase in LF will increase drainage and lower the EC in the irrigation drainage and soil salinity, and vice versa. This inverse relation is shown in Table 6.

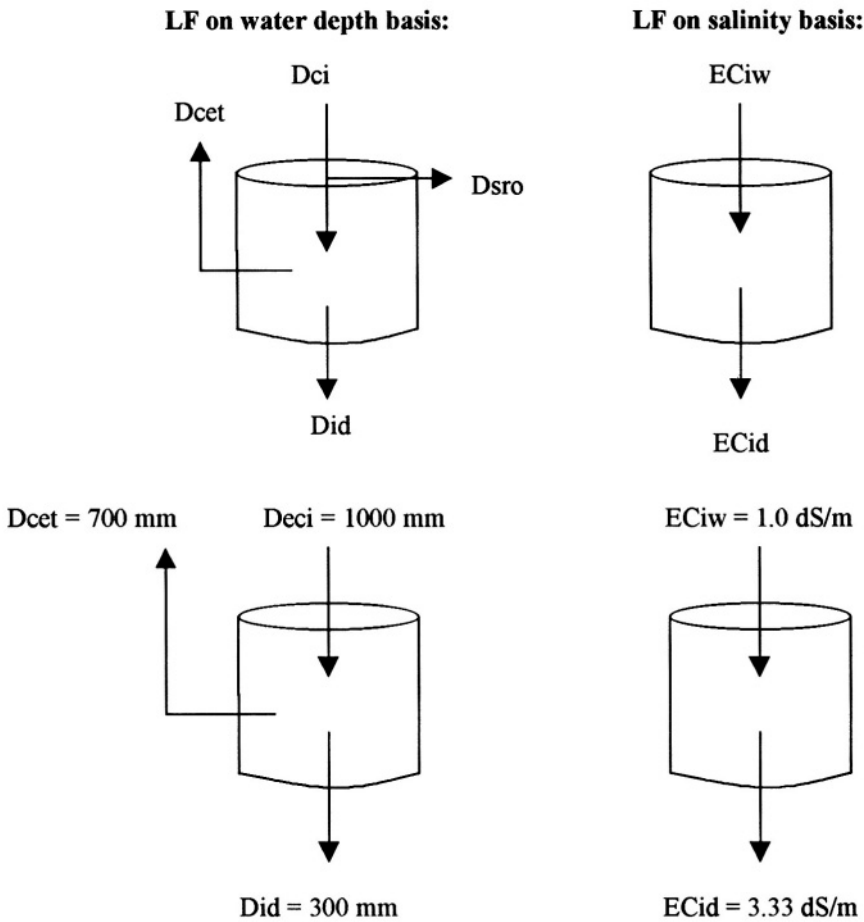


Figure 6. Leaching fraction (LF) on a water depth and water salinity basis. Dci, Dsro, Dcet, Did and Dcet, respectively denote depths of crop irrigation, surface runoff, crop evapotranspiration, irrigation drainage and effective crop irrigation while ECiw and ECid, respectively, denote electrical conductivities of irrigation and drainage waters.

Many crop plants have a rooting system by which their ET is met by a root water extraction pattern as shown in Figure 7. Here, 40% of ET is extracted from the top quartile of the rootzone, 30% from the second quartile, 20% from the third quartile, and 10% from the bottom of the root zone to satisfy ET. This 40-30-20-10 pattern is assumed regardless of the differences in depth of rooting (Rauschkolb and Hornsby, 1994). A deep-rooted tree will have the same quartile extraction pattern as a shallow rooted vegetable crop with a 40-30-20-10 pattern. Taking advantage of this generalized root water extraction pattern, a multi-layer root-zone salinity model can be readily constructed.

The seasonal LF for the bottom of each of the rootzone quartiles can be calculated with

$$LF_q = \frac{D_{eci} - \sum W_q D_{cet}}{D_{eci}}$$

(14)

where LF_q is the LF of quartiles 1, 2, 3 and 4 and W_q is the cumulative root water extraction fraction. It is assumed that the LF at the surface of quartile, LF_0 is 1.00 and there is no rootwater extraction. Table 7 gives illustrative calculations for LF of each quartile; assuming that D_{eci} is 1,000 mm and D_{cet} is 700 mm as in the previous example.

Equation (13) can be used to calculate EC_{id} in the bottom of each quartile, EC_q , by substituting LF with LF_q . Table 7 gives the corresponding EC_q for each quartile. So with these simple equations, it is possible to generate a salinity profile for lands irrigated by surface irrigation methods. The rootzone salinity profile calculated is the long-term steady-state salinity. Note that soil parameters such as soil water content and hydraulic conductivity are not considered in this simple model.

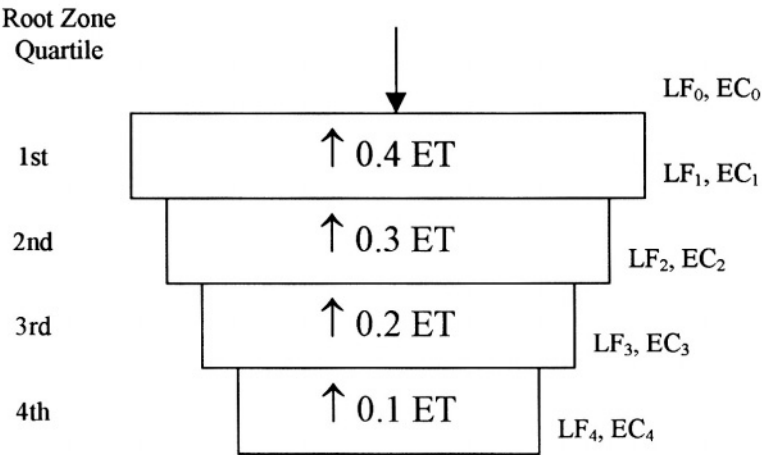


Figure 7. A schematic diagram of quartile rootzone salinity model.

TABLE 7. LF and EC by rootzone quartiles for surface irrigation.

Rootzone quartile	$LF_q = \frac{D_{eci} - \sum W_q D_{cet}}{D_{eci}}$	$EC_q = \frac{EC_{iw}}{LF_q}$
0th surface	$LF_0 = \frac{1000mm - (0)700mm}{1000mm} = 1.0$	$EC_0 = \frac{1.0dS/m}{1.0} = 1.0dS/m$
1st	$LF_1 = \frac{1000mm - (0.4)700mm}{1000mm} = 0.72$	$EC_1 = \frac{1.0dS/m}{0.72} = 1.39dS/m$
2nd	$LF_2 = \frac{1000mm - (0.7)700mm}{1000mm} = 0.51$	$EC_2 = \frac{1.0dS/m}{0.51} = 1.96dS/m$
3rd	$LF_3 = \frac{1000mm - (0.9)700mm}{1000mm} = 0.37$	$EC_3 = \frac{1.0dS/m}{0.37} = 2.70dS/m$
4th bottom	$LF_4 = \frac{1000mm - (1.0)700mm}{1000mm} = 0.30$	$EC_4 = \frac{1.0dS/m}{0.30} = 3.33dS/m$

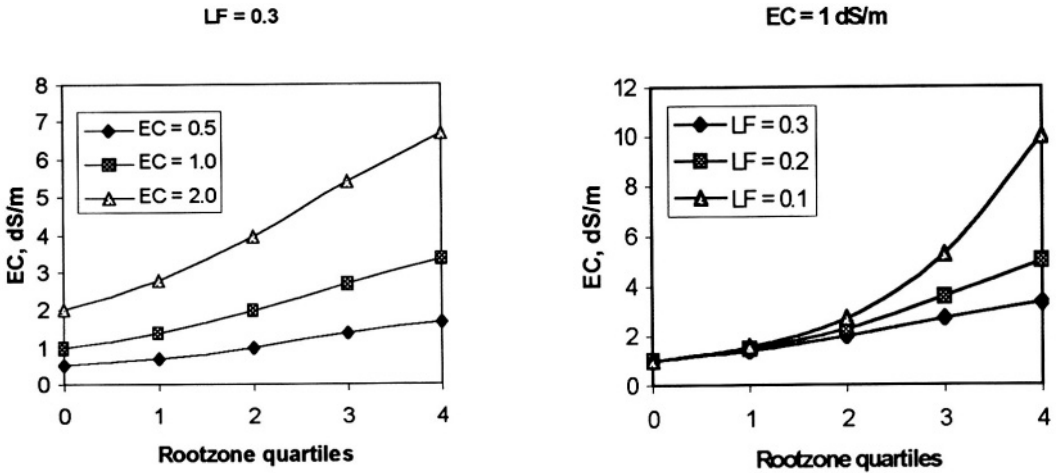


Figure 8. Sensitivity analyses on EC of irrigation water and LF for surface irrigated rootzone salinization

Figure 8 shows sensitivity analyses, changing one variable and keeping the others constant and note the differences in outputs, for quartile rootzone salt accumulation for varying LFs and EC_{iw}s. They show that in surface irrigated lands the upper part of the rootzone is the zone of leaching because quartile LFs are greater, while the lower part of

TABLE 8. LF and EC by rootzone quartiles for subsurface irrigation.

Rootzone Quartile	$LF_q = \frac{D_{eci} - \sum W_q D_{cet}}{D_{eci}}$	$EC_q = \frac{EC_{iw}}{LF_q}$
4 th bottom	$LF_4 = \frac{1000mm - (0)700mm}{1000mm} = 1.0$	$EC_4 = \frac{1.0dS/m}{1.0} = 1.0dS/m$
3rd	$LF_3 = \frac{1000mm - (0.1)700mm}{1000mm} = 0.93$	$EC_3 = \frac{1.0dS/m}{0.93} = 1.08dS/m$
2nd	$LF_2 = \frac{1000mm - (0.3)700mm}{1000mm} = 0.79$	$EC_2 = \frac{1.0dS/m}{0.79} = 1.27dS/m$
1st	$LF_1 = \frac{1000mm - (0.6)700mm}{1000mm} = 0.58$	$EC_1 = \frac{1.0dS/m}{0.58} = 1.72dS/m$
0 th surface	$LF_0 = \frac{1000mm - (1.0)700mm}{1000mm} = 0.30$	$EC_0 = \frac{1.0dS/m}{0.30} = 3.33dS/m$

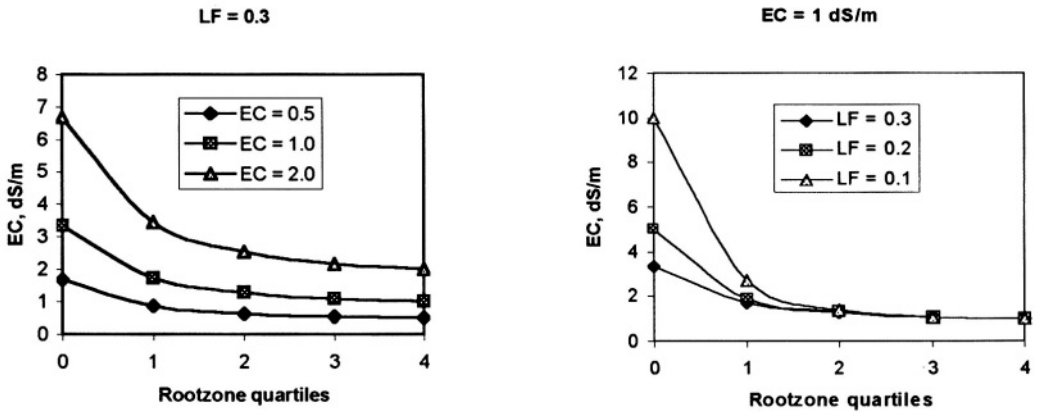


Figure 9. Sensitivity analyses on EC of irrigation water and LF for subsurface irrigated rootzone salinization.

the root zone is the zone of salt accumulation because quartile LFs are smaller. Fortunately, with a 40-30-20-10 root-water extraction pattern, the plant roots absorb less water from the highly saline bottom quartiles, an important consideration in crop salt tolerance to soil salinity levels.

This conceptual model may be also applied to subsurface irrigation in which the crop ET is entirely satisfied by sub-irrigation or shallow water table by letting irrigation take

place from the bottom. Table 8 presents the calculations. The LFs in the rootzone quartiles under sub-irrigation is bottom upwards and Wq differs slightly going from the bottom upwards as compared to top downwards. With surface irrigation, drainage out of the bottom of the rootzone as a function of LF is conceptually clear. But for sub-irrigation, drainage from the soil surface is not so clear to conceptualize. We shall, however, assume that surface drainage takes place somehow.

Figure 9 shows sensitivity analyses on quartile ECs by varying LFs and ECiws. Under sub-irrigation, salts accumulate near the soil surface rather than at the bottom of the rootzone as in surface irrigation. Such a salt distribution is not conducive to plant growth because rootwater extraction tends to take place in the zone of highest salinity. If the salt concentration is very high, however, the solute potential may add to the pressure potential such that water in surface quartiles may be less available to plants.

This model has been extended to consider initial soil salinity and monthly irrigation to predict rootzone salt accumulation but is not presented herein. The extended model simulates the shorter-term transient conditions.

2.4.3 POTENTIAL IMPACTS OF ROOTZONE SALINITY ON CROP YIELDS

Hoffman (1990) has proposed an equation to evaluate the potential impact of average soil salinity on yields of crops using the Maas salt tolerance tables (1990) with

$$Yr = 100 - b(ECe - a) \quad (15)$$

where **Yr** is relative crop yield (%), 100 is the maximum yield, “b” is the slope of declining yield with increasing ECe (% yield reduction per ECe in dS/m), ECe is the average rootzone salinity (dS/m) and “a” is the threshold ECe (dS/m) above which yield declines. Table 9 gives the salt tolerance of representative crops in which values for parameter “a” and “b” is given (see also Chapter 1, Table 4).

Equation (15) will be applied to several crops taking the EC profiles generated in the quartile salinity model in Tables 7 and 8. Since equation (15) utilizes an average rootzone ECe, Tables 10 and 11 show the calculations for obtaining arithmetic mean ECe and root-weighted mean ECe from the four quartiles. Recall that quartile ECe is the ECe in bottom of the quartile and they need to be converted to mid-depth ECe for each quartile. The results show that there are some differences between arithmetic and root-weighted mean ECe, and also some differences between surface and subsurface irrigated root zones.

For onions (*Allium cepa*), a salt-sensitive crop, the calculated Yr using arithmetic mean ECe for surface irrigated profile (3.71 dS/m) is 60% and for sub-irrigated (2.67 dS/m), 76%. In contrast, the calculated Yr using the root-weighted mean ECe for surface irrigated profile (2.65 dS/m) is 77%, and for sub-irrigated (3.50 dS/m), 63%. Corn (*Zea mays*), a moderately sensitive crop, would have a Yr using arithmetic mean ECe for surface irrigated profile of 76% and for sub-irrigated, 88%. In contrast, the calculated Yr using the root-weighted mean ECe for surface irrigated profile is 83%, and for sub-irrigated, 72%. Tall fescue (*Festuca elatior*), a moderately tolerant grass, and barley

(*Hordeum vulgare*), a salt tolerant grain, both would have Yr of 100% for all cases, since equation (15) applies only when parameter "a" is smaller than ECe. A suggestion is made that impact of root-zone salinity on crop yields be evaluated on root-weighted mean ECe basis.

TABLE 9. Salt tolerance ratings of representative crops (Maas, 1990): $Y_r=100-b(EC_e-a)$, where 'a'= threshold ECe and 'b'= slope

Crop	Coeff. a	Coeff. b	Ratings
	dS/m	% per dS/m	
dry bean	1.0	19.0	Sensitive (S)
onion	1.2	16.0	S
orange	1.7	16.0	S
peach	1.7	21.0	S
corn	1.7	12.0	Moderately sensitive (MS)
alfalfa	2.0	7.3	MS
potato	1.7	12.0	MS
rice	3.0	12.0	MS
red beet	4.0	9.0	Moderately tolerant
ryegrass	5.6	7.6	MT
zucchini squash	4.7	9.4	MT
tall fescue	3.9	5.3	MT
barley	8.0	5.0	Tolerant (T)
cotton	7.7	5.2	T
date palm	4.0	3.6	T
wheat	8.6	3.0	T

TABLE 10. Arithmetic and root-weighted mean soil salinity for surface irrigated soil with ECiw of 1 dS/m and LF of 0.1

Quartile	Quartile ECe	Arithmetic average ECe	Extraction fraction, Wq	Root-weighted average ECe
	dS/m	dS/m		dS/m
0	1.0	1.18	x 0.43	0.48
1	1.37	2.04	x 0.3	0.61
2	2.70	3.98	x 0.2	0.80
3	5.26	7.63	x 0.1	0.76
4	10.0			
Ave		3.71		2.65

TABLE 11. Arithmetic and root-weighted mean soil salinity for subsurface irrigated soil with EC_{iw} of 1 dS/m and LF of 0.1

Quartile	Quartile ECe	Arithmetic average ECe	Extraction fraction, W_q	Root-weighted average ECe
	dS/m	dS/m		dS/m
0	10.0			
		6.35	x 0.4	2.54
1	2.70			
		2.04	x 0.3	0.61
2	1.37			
		1.23	x 0.2	0.25
3	1.10			
		1.05	x 0.1	0.10
4	1.0			
Ave		2.67		3.50

Leaching Requirement (LR), a crop-specific parameter, may be used to manage rootzone salinity to maintain yields. From observations of field data, accounting for idealized salt accumulation curve, the empirical derivation of LR (Rhoades, 1972) is

$$LR = \frac{EC_{iw}}{(5 * EC_e - EC_{iw})} \quad (16)$$

For example, if EC_{iw} is 0.8 dS/m and threshold EC_e for 100% yield of barley is 8 dS/m, then $LR = (0.8)/(5(8.0-0.8)) = 0.016$. But if the crop is onion which has a threshold EC_e of 1.2 dS/m for 100% yield, then $LR = (0.8)/(5(1.2-0.8)) = 0.15$.

The depths of infiltrated water needed to satisfy both ET and LR is

$$Deci = \frac{ET}{(1 - LR)} \quad (17)$$

For barley, $Deci = (560 \text{ mm})/(1.0-0.016) = 569 \text{ mm}$ and $Did = 9 \text{ mm}$. For onions, $Deci = (430 \text{ mm})/(1.0-0.15) = 506 \text{ mm}$ and $Did = 76 \text{ mm}$.

A more comprehensive evaluation considering ET, LF, LR, and IE (irrigation efficiency) can be made by

$$LF = \left[1 - \left(\frac{IE}{100} \right) (1 - LR) \right] \quad (18)$$

Assuming an IE of 80%, for barley, $LF = [1.0 - (80/100)(1.0-0.016)] = 0.21$, and for onions, $LF = [1.0 - (80/100)(1.0-0.15)] = 0.32$.

Table 12 summarizes the above estimates. Water management to grow barley vs. onions will differ considerably with onions using more water to maintain Yr of 100% because it is salt sensitive. Salt balance in the rootzone to maintain crop yields is strongly influenced by water management.

TABLE 12. Summary of water management to satisfy ET and LR of onions and barley utilizing a 0.8 dS/m EC water at 80% irrigation efficiency.

Parameter	Onion	Barley
Crop ET, mm	430	560
Threshold salinity, ECe	1.2	8.0
LR	0.15	0.016
Irrig. drainage for LR, mm	76	9
Irrig. efficiency, %	80	80
Surface runoff, mm	162	119
Total applied irrig., mm	668	588

2.4.4 LAND RECLAMATION OF SALINE AND SODIC SOILS

Soils suffering from either primary or secondary salinization may be reclaimed to increase their productivity. Saline soils are easier to reclaim than sodic soils because the former requires leaching of soluble salts while the latter requires a Ca source to replace excess ESP in addition to leaching. But many saline soils exist under high water table conditions so that subsurface drainage needs to be installed first.

In addition, the soil should be permeable for leaching. If the soil requires improvements in soil permeability, tillage, ripping clay and hard pans or soil/water amendments may be needed. Reclamation leaching for soluble salts is accomplished with waters of lower salinity than the salt-affected soil and/or the desired salinity level to meet the threshold salinity of the intended crop. Leaching soluble salts from the top 40 to 60 cm is usually adequate for most crops (Keren and Miyamoto, 1990). The drainage water produced may be quite saline and its disposal could be a problem. Leaching of saline soils can be carried out by continuous or intermittent ponding. The former carried out under near saturated soil-water conditions usually takes more water than the latter which is carried out under unsaturated conditions. Hoffman (1980) has proposed a salt-leaching efficiency equation with

$$\frac{C}{C_o} = \frac{k}{[D/D_s]} \tag{19}$$

where C is the soil salinity after leaching, Co is the soil salinity before leaching, D is the depth of leaching water applied, Ds is the depth of soil to be leached, and k is an empirical coefficient that ranges from 0.1 for sandy loam to 0.3 for clay under continuous ponding. Equation (19) can be used to calculate the depth of water needed to

leach salts from a desired depth of soil for specified initial salinity and desired salinity. Figure 10 shows plots for continuous and intermittent leaching using the relationship of C/C_0 , fraction of initial soil salinity remaining, and D/D_s , depth of leaching water to unit depth of soil. The leaching efficiency is affected among others by pore size distribution in the soil. The value of parameter k under intermittent ponding conditions or by intermittent ponding or sprinkling is about 0.1 for all mineral soils because unsaturated flow of water and salts takes place in smaller pore sizes.

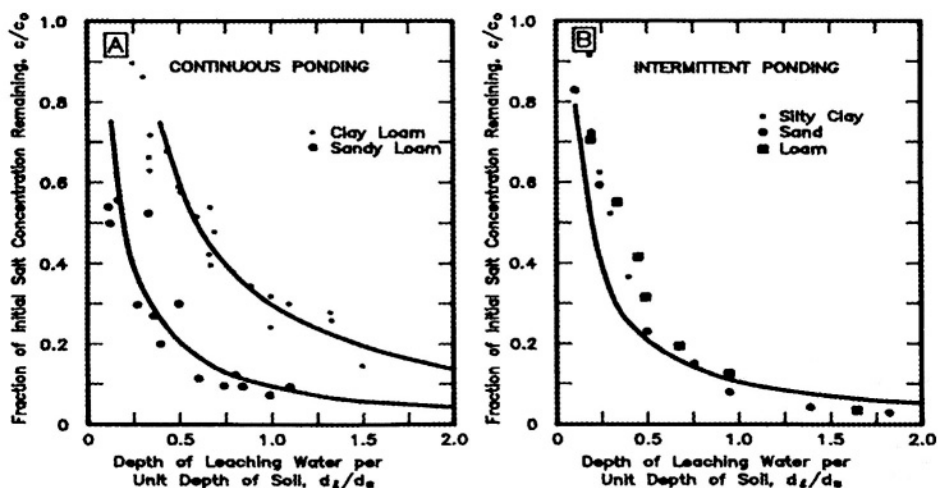


Figure 10. Salt leaching under continuous and intermittent ponding of saline soils (Hoffman, 1980)

The reclamation of sodic soils requires replacement of excess ESP with soluble Ca (the reverse reaction of equation (9)). Various Ca sources are available including soil gypsum and calcite and soil/water amendments such as gypsum. The dissolution of gypsum and the exchange between Ca^{2+} and exchangeable Na will result in production of salines and they will have to be leached out of the rootzone. If a low solubility Ca mineral is present in the soil such as calcite, which is usually the case in arid zone soils, addition of acids (e.g., sulfuric) or acid forming amendments (e.g., elemental sulfur) will dissolve calcite and generate soluble Ca^{2+} . Another slower approach of reclaiming sodic soils is the cultivation of paddy rice in which CO_2 in the soil solution is increased, making calcite more soluble, and the Ca^{2+} generated aids in reclaiming the sodic soil.

One of the earliest computer-based simulations of land reclamation of saline and sodic soils was utilized by Tanji *et al.* (1972) for chemically stratified soil profiles. This model linked a soil chemistry sub-model to a chromatographic salt transport sub-model to simulate the concentrations of major ions, sulfate ion pairing, solubility of gypsum and cation exchange. The model predicts the depth of water and gypsum amendment, if needed, to reclaim the soil to target salinity and sodicity levels to a given soil depth.

2.4.5 NUMERICAL SIMULATION MODELS OF SALT TRANSPORT

Presently, there are a number of numerical transient models that simulate soil salinization and salt leaching in cropped soil profiles in one and two dimensions. The 1-D models include LEACHC (Hutson and Wagenet, 1992), SOWACH (Dudley and Hanks, 1991) and UNSATCHEM (Suarez and Simunek, 1995). The 2-D models include HYSAM (Nour El Din *et al.*, 1987 and Karajeh and Tanji, 1994) for tile- drained cropped systems. These numerical models couple a water-flow sub-model, Richard's equation with a rootwater extraction term, and a soil chemistry sub-model with sink/salt terms (mineral solubility, ion association and cation exchange). The partial differential equations in the model are solved by finite difference or finite element methods, taking small steps in time and space. These models require numerous input data and model coefficients and therefore can be validated with only heavily monitored research plots. Suarez and Dudley (1998) made a comparative performance of LEACHC, SOWACH and UNSATCHEM. They conclude that the models greatly enhance our understanding of detailed interactive processes but are difficult to validate under field conditions and have differing model assumptions and sensitivities, especially carbonate chemistry, and direct comparisons between models are difficult. They also conclude that plant water uptake strongly influences water and solute movement.

2.5 Cutting Edge Research and Contemporary Problems in Soil Salinity

One of the most exciting modeling research being carried out is the coupling of 3-dimensional transient modeling of root growth and root water and nutrient uptake simultaneously with soil water and solute transport (Somma *et. al.*, 1998). Such a modeling effort substantially increases our understanding of the complex interactions taking place in the soil-plant-atmosphere continuum. The advances made in modeling and visual conceptualization show considerable promise for fruitful interactions between plant scientists and numerical modelers.

Although paddy rice has been used to reclaim sodic soils world wide, the temporal and spatial aspects of CO₂ production and the rate at which sodic soils are reclaimed have not been fully addressed. It appears that considerable advancements could be made if the usual change in soil hydraulic conductivity with changing SAR/ESP and electrolyte content could be simulated.

The disposition of saline drainage water into the environment from salt- and drainage-impacted lands is a growing problem especially when the drain water contains constituents such as selenium that is toxic to wildlife and fish (Tanji *et al.*, 1986; National Research Council, 1989; San Joaquin Valley Drainage Program, 1990). FAO and ICID (International Commission on Irrigation and Drainage) have recently published a report on the management of agricultural drainage water quality (Madramootoo *et al.*, 1997). Constraints on the discharge of saline waters result in salt imbalance in cropped lands. Furthermore, marginal quality irrigation waters are increasingly being used and/or reused in agriculture (Tanji, 1997). These marginal quality waters include saline waters, irrigation drainage (Grattan, 1994), treated sewage effluents (Asano, 1994), wastewaters from food processing plants and confined animals.

The long-term use of such marginal quality waters on soil properties and plant performances needs to be assessed.

2.6 Summary

The performance of plants is affected by soil salinity. Since actively transpiring plants evapoconcentrate soil solutions and soil water moves in the direction of hydraulic gradient, plants are continuously subjected to ever changing soil moisture and salinity. In turn, soil salinity is a non-conservative parameter because it participates in sink and source mechanisms. Moreover, plant uptake of nutrients and other solutes may be affected by chemical speciation in the soil solution. Water management is a key to salinity control in irrigated agriculture. This chapter written by a soil scientist is intended to provide plant scientists with a background in soil salinity with a focus on irrigated soils.

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CHAPTER 3

SALINITY, HALOPHYTES AND SALT AFFECTED NATURAL ECOSYSTEMS

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Abstract

A high diversity of halophytes has evolved in predominantly arid regions. The mechanisms of salt resistance are manifold. Classification of halophyte types can be done by using various parameters. Ion ratios play an important role in adaptation of halophytes. Succulence is a special feature of halophytes, enhanced by salts in stem- as well as in leaf-succulents. Recretion of salts by salt-glands or bladders is another mechanism to cope with salinity. Natural saline ecosystems are occurring worldwide along coasts, but at inland sites are created predominantly in arid regions.

3.1 Introduction

Potential evaporation in arid regions is higher than precipitation. Rainwater contains ions and run-off water is leaching rocks and soils. This leads inevitably to the accumulation of salts in soils, especially in the lower areas around the erosion basins. With the use of irrigation water the processes are the same but are much faster.

Erosion basins of the arid regions are thus saline and constitute the proper habitat for halophytic vegetation. This is characteristic of most of the arid endorrheic basins. Soils of irrigated agricultural fields of those regions exhibit a rather fast accumulation of salts.

Effects of salinity on the natural vegetation types are on various levels. Some 5% of the European flora are halophytic, whereas in the Iranian flora they reach some 7%. Such plants constitute the various halophytic vegetation types.

Various types of salinity are known (chloride-, sulphate-, carbonate-, magnesium-, and boron-), depending on soil properties (Chapter 2), climatic conditions and ecosystem processes. The presence of excessive ions in such ecosystems dominates over many other environmental factors, and leads to an overall similar physiognomy of saline vegetation types, to a great extent irrespective of climate.

3.2 Mechanisms of salt tolerance in halophytes

3.2.1 TOLERANCE AND RESISTANCE

Plants are subjected to a variety of ecological factors, varying with space and time. It is generally accepted that tolerance to a specific ecological factor such as drought, frost, heat, salinity etc. is the more specific term to describe a distinct adaptation of a plant species in coping with the relevant stress-factor, whereas resistance is the overall general term (Waisel, 1972; Levitt, 1980). Salt resistance has different components. At the whole plant level it may be the process of salt regulation, but at the cellular level it may be the salt tolerance of the cytoplasm (Popp, 1985).

Plants growing naturally on saline stands have evolved various mechanisms to cope with salinity. Table 1 gives examples of control mechanisms in plants for maintaining a rather constant level of sodium concentration in the living plant tissues. This is regarded one of the major factors of adaptation in halophytes in contrast to that of non-halophytes ("glycophytes") besides of osmotic adaptation (see below).

TABLE 1. Control mechanisms of halophytes to strive on saline sites (Breckle, 1990)

A Avoidance	
a	growth only during favourable seasons (time niche)
b	growth only on favourable sites (site niche)
c	limitation of root growth and absorption activity to distinct soil horizons (site niche)
B Evasion and adaptation processes	
a	selectivity against Na and Cl
b	leaching of salt from shoots
c	diversion of salt out of assimilating tissues
d	compartmentation of salt within plant, within tissues, within cells
e	accumulation of salt in xylem parenchyma in roots and shoots
f	synthesis of organic solutes
g	retranslocation of salt to roots and recretion by roots
h	disposal of older plant parts ("salt-filled organs")
i	recretion by gland-like structures on shoots
	1 by salt-glands
	2 by salt-bladders
C Tolerance	
a	increasing salt tolerance of tissues, cells, organelles
b	increase in halo-succulence
	1 increase in leaf-succulence
	2 increase in stem-succulence, reduction of leaves

Salt resistance of plants can be defined in various ways. Some of these control mechanisms are also active in non-halophytes (Tables 1 and 2, Figure 1), but some of them are very specific features, which have evolved in halophytes. Moreover, it is obvious that not only one of these mechanisms is active in different types of halophytes. One set can be active in one group, whereas another set of mechanisms is dominant in another group. This has lead to the classification of halophytes into salt-excluders, salt-includers (often possessing means of salt-recretion) and salt-accumulators (often halosucculents).

It has to be stressed that salt resistance has at least two quite differing aspects: The one is the upper limit of salt that can be tolerated by an individual plant, the other is the existence of a plant species that exerts successful reproduction. Salt resistance of plants varies very much. It varies

- during different growth or development phases
- with ionic constitution of the soil solution
(e.g. the presence of Ca, K as antagonists of Na)
- with microclimatic conditions (e.g. relative humidity)
- with life form and halophyte type
- with the plant organ affected by salinity
- with the genetic variability of each species forming ecotypic varieties

TABLE 2. Salt resistance of plants (acc. to Richards, 1954; Waisel, 1972; Breckle, 1976; Albert, 1982)

A Ecological Aspect:

The ability of plants to survive and to complete their life-cycle under saline conditions
☐ determines survival ability and endurance; criterion for wild species;
growth, size, yield, are neglected

B Physiological and Ecophysiological Aspect:

The relative ability of a species to grow on saline soils in comparison to its growth on nonsaline, conditions
☐ determines adaptabilities of species more than their ultimate resistance

C Agricultural Aspect:

The relative ability of a species to grow and yield on saline soils in comparison to other species
☐ determines yield

Salt tolerance of a plant is not defined by the act of individual genes, by the individual regulation of each of them or by one specific metabolic process, salt tolerance is a whole plant response (Hedenström and Breckle, 1974; Breckle, 1990, 1995; Munns, 1993; Naik and Widholm, 1993; Flowers and Yeo, 1995; Ramani and Apte, 1997), where many processes, such as efficient K-pumping and accumulation, synthesis and transport of compatible solutes, plant signaling systems involved in tissue and in developmental regulation (Winicov and Bastola, 1997) etc., are only some important adaptations.

3.2.2 MULTILEVEL EFFECTS OF NaCl

Salt affects plants in various ways and on different complexity levels. There are the osmotic effects (Albert, 1982; Sitaramam and Madhavarao, 1997; Pardossi *et al.*, 1998) and the specific ion effects to be distinguished (Neumann, 1997). Both affect in different ways the cytoplasmic structure and metabolic processes. On the level of the individual plant these processes are underlying genetic and hormonal control. Thus formative effects are genetically determined as well as modificatively influenced (e.g. Suarez *et al.*, 1998). On the level of a plant community the ecological success, which is influenced by dry matter production, carbon partitioning, growth and seed set, may alter the competitive ability of a species. Under field conditions, i.e., under competition with other species, and under varying abiotic and biotic factors, a distinct pattern of dominance in a plant association develops. Selection of more resistant ecotypes may lead to an altered composition and in the long run change the community structure.

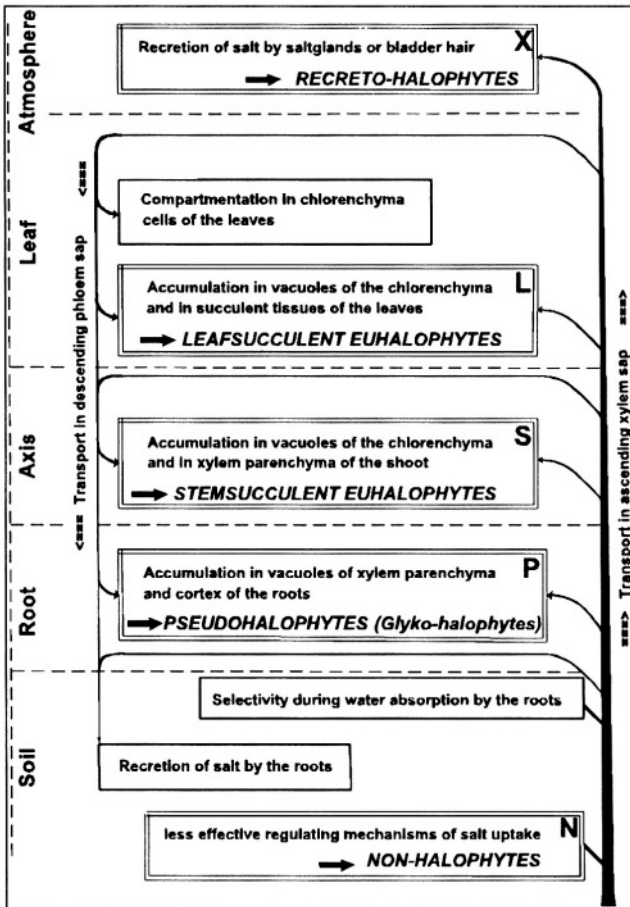


Figure 1. Schematic classification of halophyte types according to strategy of regulation of internal salt concentration (Breckle, 1976)

3.3 Halophyte types

3.3.1 CLASSIFICATION

Halophytes can be grouped to a specific halophyte-type, according to most relevant mechanisms for salt resistance (Figure 1). However, since intensive halophyte ecology research has started (Stocker, 1928; Waisel, 1972), there were long discussions how to classify halophytes and many classification systems had been proposed (Breckle, 1990).

It is most promising to use the ecophysiological approach in defining halophyte types. Thus, as demonstrated in Figure 1, halophyte types can be characterized by the dominance of processes regulating salt transport in plant organs according to accumulation and recretion of salts, thus maintaining a distinct saltbalance.

Most of the halophytic genera can be placed distinctly in one of these groups (Figure 1). Very few exhibit intermediate characters or change from one to another category with age (e.g. *Halimione*).

For halophytes it is characteristic that osmotic adaptation is accomplished by absorbing inorganic ions (expressed as Cl^- in Figure 2), counterbalanced by compatible solutes in the cytoplasm. As a rule the osmotic potential of leaf cell sap differs normally by 0.5 to 1 MPa from that of the soil solution (Figure 2), enabling uptake of water.

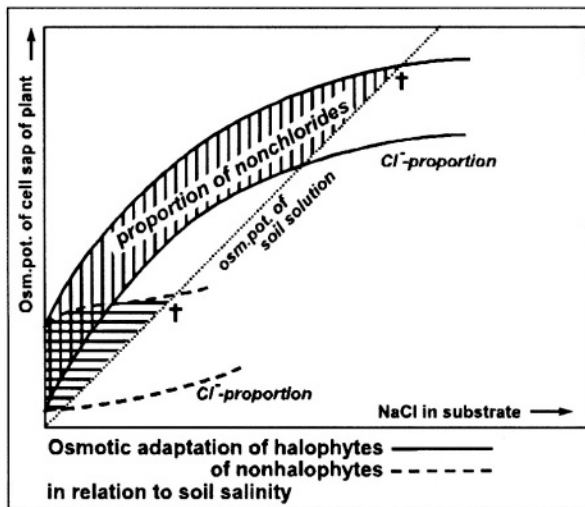


Figure 2. Osmotic adjustment in halophytes and nonhalophytes (□: maximum NaCl concentration, where osmotic adaptation is zero and thus plants start suffering)

3.3.2 GROWTH OF HALOPHYTES

In many halophytes it was shown that growth is stimulated by salt. According to growth response curves Kreeb (1974) has distinguished four plant types, as is shown in

Figure 3. Halophytes were defined those which exhibit maximal relative growth under some salinity in the substrate.

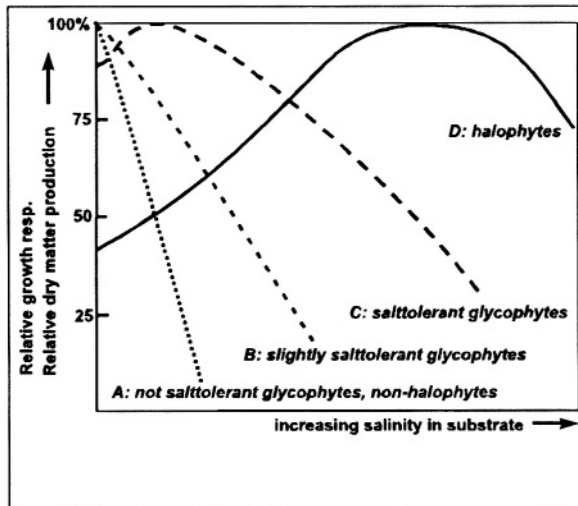


Figure 3. Schematic graph indicating growth of various types of plants under salt-stress (modified from Kreeb, 1974)

However, it has not been taken into account, if growth of the root system and/or the shoot is equally affected. An increased shoot/root ratio with increased salinity was shown by Osmond (1980) for *Atriplex*-species. But Clarke and Hannon (1970) reported a distinct decrease of shoot/root ratio with increasing salinity for a number of Australian saltmarsh plants, Shalhevet *et al.* (1995) for maize and soybean. It should be mentioned that other ecological factors, such as, nitrogen availability can greatly change growth. Moreover, under drought stress salinity enhanced growth in various *Atriplex*-species (Glenn and Brown, 1998), indicating an important role of ion uptake in halophytes.

Roots are in direct contact with the saline soil solution. Salinization results in shorter and thicker roots (Cramer *et al.*, 1991) and it seems again, that not only osmotic effects but also specific ion effects cause an additional depression of growth (Kafkafi and Bernstein, 1996). A change in architecture of the root system under salinity can be deduced from results of Waisel and Breckle (1987). They have shown that the laterals are more affected by NaCl than the taproot. A horizontally spread root system under nonsaline conditions develops to a more vertically oriented root system under salinity. Unequal distribution of salts in the root environment was investigated by Sonneveld and Kreij (1999). Nutrients were readily absorbed also from roots under high salinity, whereas water was preferably absorbed from the root parts with the lowest salinity.

In various chenopods it was shown that growth was stimulated by moderate salinity (Breckle, 1976). The best way to define growth is using ash free dry matter (organic matter) increment, which often in literature is not reported. Plant mineral content in

halophytes may be of such magnitude that changes in organic matter could be obscured.

Since growth in halophytes is enhanced by salinity in contrast to nonhalophytes, this leads to the question whether halophytes need salt obligatorily. As obligatory halophytes only would thrive successfully on saline soils the question arises if they can live on nonsaline soils. It has been shown that sodium is an essential micronutrient only in C4- and in CAM-plants (Brownell, 1979 – Chapter 16), but deficiency symptoms are known only under experimental conditions. The same is known for chloride (Epstein, 1980, 1985; Engel *et al.*, 1997). There are no indications that any saltmarsh or mangrove species is an obligate halophyte, they all can be grown successfully under garden cultivation, but if they also can complete their whole life-cycle successfully under such conditions it was not proven. The effects of salinity on different growth stages and growth processes of plants also have to be taken into account (Ungar, 1996). Germination and seedling growth is normally more sensitive than growth of established adult plants. Köhl (1997) has shown that ecotypic differentiation of closely related salt marsh and inland taxa of *Armeria maritima* is not very significant in respect to salt tolerance though it is rather obvious for heavy metal resistance. Halotolerance depends largely on the osmotic adjustment of cytoplasm through accumulation of compatible solutes (Wyn-Jones *et al.*, 1977; Popp, 1985; Shomer-Ilan and Waisel, 1986; Solomon *et al.*, 1994; Gzik, 1996; Hare and Cress, 1997). Screening for salt resistance must be based on a good knowledge of the involved traits and on understanding of the internal metabolism in plants with an appropriate content of organic protectants (Waisel 1989).

Salts taken up by halophytes do not directly control plant growth by affecting turgor, photosynthesis or the activity of one or another enzyme. The build-up of salts in old leaves hastens their death. The loss of leaves affects the supply of assimilates or hormones to the growing organs and hereby affects growth (Munns, 1993; Munns *et al.*, 1995). As a conclusion certain forage crops might be cultivated by irrigation with brackish water without any yield reduction for a shorter period but not with seawater (Pasternak *et al.*, 1995). Contamination of soils with sodium should be avoided in any agricultural system. Break-down of physical soil structure by sodium leads inevitably to growth reductions.

3.3.3 ION-RATIOS

Halophytes can be classified into Chloride-Halophytes, Sulfate-Halophytes and Alkali-Halophytes, according to the main ions in cell sap or ash (Walter, 1968). The Alkali-Halophytes are those, where a high proportion of organic acids (e.g., oxalate for example in *Halogeton* with up to 30% of dry matter) are accumulated. It is long known that halophytes are able to take up nutrients from the soil despite of an excessive content of Na^+ and Cl^- . Most halophytes are discriminating Na^+ against K^+ and only few species are sodiophilic. To demonstrate the characteristics in K^+/Na^+ -discrimination it is necessary to have the relevant soil samples from the rhizosphere. Various halophytes from Iran and Afghanistan were analyzed and K^+/Na^+ -ratio in the

substrate and in the plants is plotted (Figure 4). The oblique lines (f) give an idea of the accumulation factor for sodium in comparison to potassium. It is easily visible that most species under a wide range of given cation ratios in the soil favour potassium uptake. Only the Chenopodiaceae *Salicornia europaea* and *Suaeda maritima* can be termed sodiophilic. In contrast the grass *Puccinellia distans* very selectively accumulates potassium by a factor of 100 even in very saline soils.

Under controlled conditions with hydroponics or sand-culture it is possible to check the ion discrimination by different halophyte types in an even better way and this leads to characteristic indicator lines according to the given ion-ratio (Figure 5). This demonstrates again that selectivity varies according to ion ratio in the nutrient solution. Under equimolar K^+/Na^+ culture solution only the stemsucculent *Allenrolfea* is sodiophilic, whereas the leafsucculent *Sarcobatus* and even more the pseudohalophyte *Ceratoides* are potassiophilic. However, under a high supply of potassium even *Ceratoides* becomes sodiophilic, and under a very high relative supply of sodium even *Allenrolfea* becomes "potassiophilic", when comparing only with the 1:1-line.

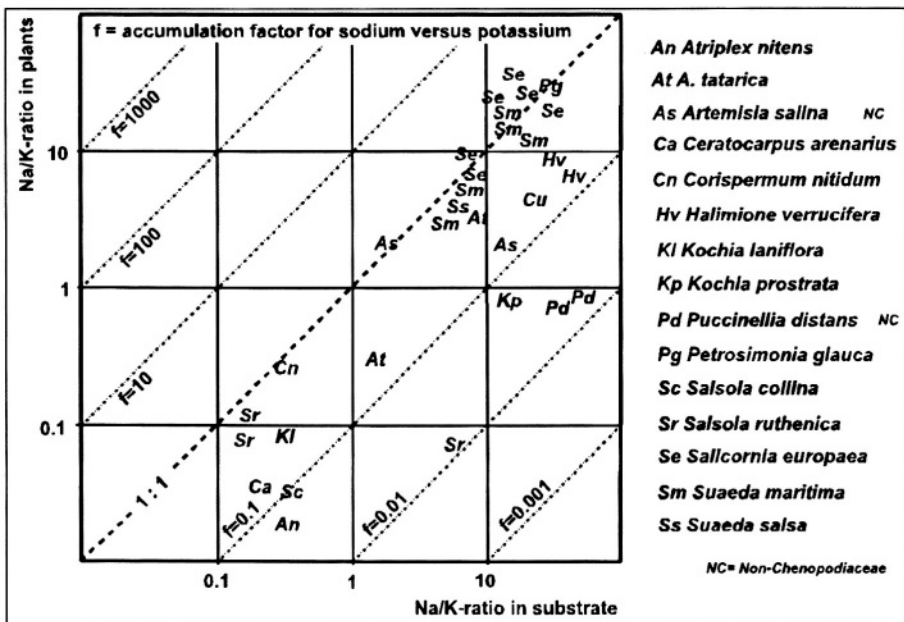


Figure 4. Ion ratio of Na/K in soil and plants indicating potassiophily (Mirazai and Breckle, 1978)

Several grades of K^+/Na^+ selectivity and sodium exclusion were demonstrated for halophytic and nonhalophytic members of the Chenopodiaceae (Reimann, 1992; Reimann and Breckle, 1993). Thus the various members of the Chenopodiaceae can not be put in one group of physiotypes. The two halophytes *Suaeda maritima* and *Atriplex prostrata* exhibited a pronounced Na^+ accumulation in the shoots.

High levels of NaCl accumulation are not necessarily a prerequisite of high salt resistance in halophytes of the salt accumulation type. Glenn *et al.* (1992) investigated low-sodium and high-sodium ecotypes of *Atriplex canescens* over a range of salinities up to 720 mol m^{-3} NaCl. Clear differences in Na^+ and K^+ -accumulation and Na^+/K^+ ratios were not paralleled by a difference in salt resistance. This means, salt resistance is more than tolerance against high internal salt concentrations. Anyhow, the capacity of plants to maintain a low cytosolic Na^+/K^+ ratio is likely to be one of the key determinants of plant salt tolerance (Maathuis and Amtmann, 1999; Chapter 8). Under natural conditions the Na-levels vary very much, but also the levels of other ions. There are many papers on the chemistry of halophytes and their internal ion composition (Albeist, 1982), as well as on the normally taxon specific accumulation of compatible solutes (Popp, 1985). Such results indicate an ion homeostasis in halophytes under NaCl stress, which partly is explained by the relevant ion transport across the cell membranes (see Niu *et al.*, 1995) of the various tissues functioning as storage cells by maintaining their internal steady state.

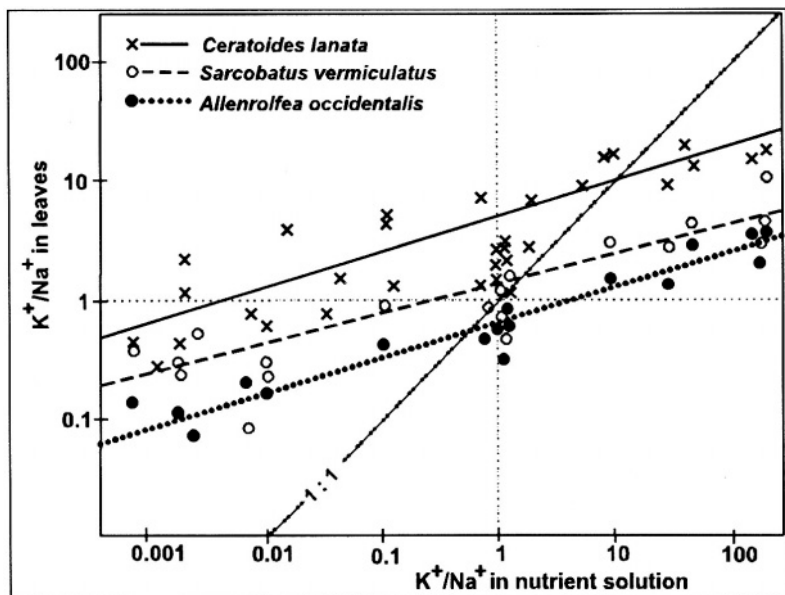


Figure 5. Ion ratios (K/Na) in nutrient solution and leaves in three North American halophytic species, differing in their potassiophily (Breckle, 1976)

Calcium has an ameliorating effect upon the tolerance of higher plants to salinity (Munns *et al.*, 1983; Epstein, 1998; Chapter 10) and the availability of Ca plays a major role in counteracting salinity stress (LaHaye and Epstein, 1969), but not in all plant organs (Caines and Shennan, 1999) and not in all growth stages (Wilson *et al.* 2000). Carvajal *et al.* (2000) could relate the ameliorative effect of Ca^{2+} to water-channel function in cell membranes of roots.

In some parts of the world salinity is not caused only by chloride, but in temperate and cold arid regions it may be by sulfate or carbonate accumulation (Curtin *et al.*, 1993). In other areas the accumulation of soluble boron compounds concomitantly with chlorides can cause high toxicity problems. Chloride and borate behave antagonistic in uptake by some halophytes (Holloway and Alston, 1992), however, Grieve and Poss (2000) found a strong interaction of external B and salinity to limit growth and yield of wheat.

3.3.4 SUCCULENT EUHALOPHYTES

Succulent halophytes are either leafless and stem-succulent or have fleshy and succulent leaves. In both cases this kind of succulence has two components, the basic one is a genetically controlled succulence, whereas the second is a modificative variable and can be induced by salts to a considerable degree. These types of halo-succulence have to be distinguished from xero-succulence.

3.3.4.1 *Leaf-succulent- Eu-Halophytes*

The typical leaf-succulent halophytes are characterized by almost cylindrical or globular leaves (examples are: *Triglochin maritima*, *Plantago maritima*, and many species of *Salsola*, *Suaeda*, *Nitraria* etc.). The salt-content of those leaves can reach up to 60% of dry matter (Eshel, 1985), the concentration of salt in cell sap about 1500 mol m^{-3} NaCl (Waisel, 1972).

The degree of succulence varies with external salinity, NaCl being more effective than sulphate-salinity. In some halophytes only some tissues in the leaves become succulent, in others all tissues grow larger and accumulate more cellsap. A higher tissue water content goes normally parallel with a lower tissue elasticity and a greater hydraulic conductance (Youngman and Heckathorn, 1992).

Some halophytes show a great increase in succulence already at low salinities. Thus, the view, that increased halo-succulence is a means for regulating ionic content of the tissues (as some kind of dilution) may be inappropriate (Storey and Wyn-Jones, 1979). It could be speculated that it might be a "cheap" possibility of increasing leaf-area for photosynthesis by low energy costs (Y. Waisel, personal comm.).

There are leaf succulent halophytes which are annuals, e.g., some *Suaeda*-species, *Haloepelis*, *Halimocnemis*, *Gamanthus* etc.. Tremblin and Ferard (1994) have shown, that *Haloepelis* has an extraordinarily high growth optimum at 200-300 mM NaCl in the medium. Other leaf-succulents are herbal perennials (such as, e.g., *Plantago*, *Aster*, *Suaeda*), and others are shrubs (e.g., some members of the genera *Salsola*, *Suaeda*, *Nitraria*, *Kochia/Maireana*). In some others the succulence of the fruit or parts of the fruit became very pronounced (*Gamanthus*, or in many Mesembryanthemaceae). Regarding the adaptations of the photosynthetic pathway, which have evolved, it is obvious that succulence has altered the anatomical structure dramatically, as can be seen in the various types, that are exhibited by *Salsola* and *Suaeda* (Shomer-Ilan *et al.*, 1981).

3.3.4.2 *Stem-succulent Eu-Halophytes*

As in leaf-halosucculents it is thought that also in stem-succulent halophytes the high salt-concentration in the plant body is mainly restricted to the large vacuoles of storage tissues cells. It was often said, that this is part of the mechanism that is responsible for diluting the salt-concentration of the salt-affected cells.

There are different lifeforms known in stem-succulent halophytes, annuals such as, e.g., *Ofaiston*, *Salicornia*, but also herbal perennials, (e.g., *Arthrocnemum*, *Anabasis*, etc.) and halfshrubs and shrubs, with woody base (e.g., *Halocnemum*, *Halostachys*, *Aellenia*, *Haloxylon*, *Kalidium*, etc.).

3.3.5 SALT-RECRETING HALOPHYTES (CRINO-HALOPHYTES)

Many halophytes exhibit a rather rapid turnover of their leaves. The rosette leaves in *Limonium vulgare* are replaced during the vegetation period 2 or 3 times, the leaves of *Aster tripolium* rather soon become yellow and new leaves replace them. This replacement is a mechanism of removal of large quantities of salt. Old leaves with high salt content are steadily replaced by younger leaves in many *Juncus* species. This is certainly one adaptation mechanism that enables the plant to get rid of excessive salts by shedding plant organs.

But even more important in some halophytes is the existence of specific cell structures which can recrete (recretion in the sense of Frey-Wissling, 1935: elimination of substances not metabolically changed) inorganic ions, especially NaCl. This is done by salt-glands, which have evolved several times in the angiosperms, and by bladder hairs. Salt glands eliminate salt to outside, bladder hairs accumulate salts in their huge vacuole, in both cases the salts are physiologically isolated from active tissues.

3.3.5.1 *Halophytes with salt glands*

Salt glands were first described by Volkens (1884) in *Limonium* and by Marloth (1887) in Tamaricaceae.

Salt glands are found in various halophytic plant genera, the main examples are listed in Table 3, exhibiting striking differences in anatomical structure. The most complex glands are the salt glands of the Plumbaginaceae (Wiehe and Breckle, 1989). The density of the salt glands is between 600 and 5000 per cm² leaf area.

The processes of salt recretion by glands were extensively studied only in a few species (see discussion in Waisel, 1972; Breckle, 1976), and it was shown that active transport plays the major role. The selectivity against ions differs in the various species according to their natural occurrence. The halophytes *Limonium gmelinii* and *L. ramosissimum* are very potassiophilic, as can be seen by the strong change in ion pattern (Figure 6, left side) between nutrient solution and leaf cell sap. Again, there is a major change in ion composition between leaf cell sap and the recreted fluid. The ion pattern changes in such a way that the cytoplasm of the leaf cells is kept relatively low in sodium, whereas the gland fluid is rich in sodium (Figure 6, right side). Such a behaviour is not recognizable in *Limonium sinuatum*, a plant which comes from

slightly saline stands. In that species selectivity in both cases of transport is low (Figure 6). It was also shown that the activity of the salt glands of the halophilic *Limonium* species (Wiehe, 1986) and *Aeluropus* have a threshold value and start to recrete NaCl only after a distinct salinity level in the leaf is reached (Pollak and Waisel, 1979).

In *Aegialitis annulata* about 26% of the internal salt content of young leaves (old leaves: 2%) is removed within 12 hours by the gland activity, when grown on 50 mol m⁻³ NaCl, but 38% (old leaves: 29%), when grown in 500 mol m⁻³ NaCl (Popp *et al.*, 1993). This is not so for *Avicennia*, where salt-filtration by the roots is by far the most important salt-rejecting mechanism (Waisel *et al.*, 1986).

TABLE 3. Halophytic plant genera possessing active salt glands

Plant family and genus		number of cells per salt gland
Convolvulaceae	<i>Cressa</i>	??
	<i>Ipomaea</i>	??
Frankeniaceae	<i>Frankenia</i>	??
Plumbaginaceae	<i>Armeria</i>	16
	<i>Limoniastrum</i>	16
	<i>Limonium</i>	16 or 20
	<i>Plumbago</i>	16
	<i>Aeluropus</i>	2
Poaceae	<i>Chloris</i>	3
	<i>Spartina</i>	2
	And many other grass genera	
	<i>Glaux</i>	??
Tamaricaceae	<i>Reaumuria</i>	??
	<i>Tamarix</i>	8
and in mangroves (Popp, 1985):		
Acanthaceae	<i>Acanthus</i>	8
Avicenniaceae	<i>Avicennia</i>	(5)-8-(9)
Myrsinaceae	<i>Aegiceras</i>	??
Plumbaginaceae	<i>Aegialitis</i>	40

Marcum *et al.* (1998) have checked 57 accessions from 5 species of *Zoysia* (grasses with two-cellular glands) for recretion of NaCl during 400 mol m⁻³ NaCl culture. Relative salinity tolerance was negatively correlated with leaf sap NaCl and positively correlated with leaf salt gland recretion rate and leaf gland density. Gland structure and function were investigated by Liphshitz and Waisel (1982).

The salt glands of *Tamarix* produce an alkaline recretum (Waisel, 1992). It is suspected, that this could promote a triplet of purposes: a) remove excess mineral ions out of the live parts of the leaves; b) recrete hygroscopic solutes, thus, providing the plants with a moist cover and reducing the time during which transpiration occurs; c) award plants with an enriched environment of CO₂, as a type of carbon enrichment system which might improve photosynthesis during early morning hours. Such an enriched environment reduces respiration and improves salt resistance of those plants.

3.3.5.2 *Halophytes with bladder hairs*

Not only glands but also bladder hairs can remove salts from essential and salt-sensitive metabolic sites in the leaf mesophyll and from the embryonic tissues close to the shoot-tip. Salt accumulating bladders are well known in several genera of the Chenopodiaceae, typical in most *Atriplex*-species (Schirmer and Breckle, 1982; Breckle, 1992). They are composed normally of one or more small stalk cells, functioning as gland cells and a huge bladder cell, functioning as a reservoir (Black, 1954, Berger-Landefeldt, 1959). The cell wall of the stalk cells seems to be largely cutinized preventing

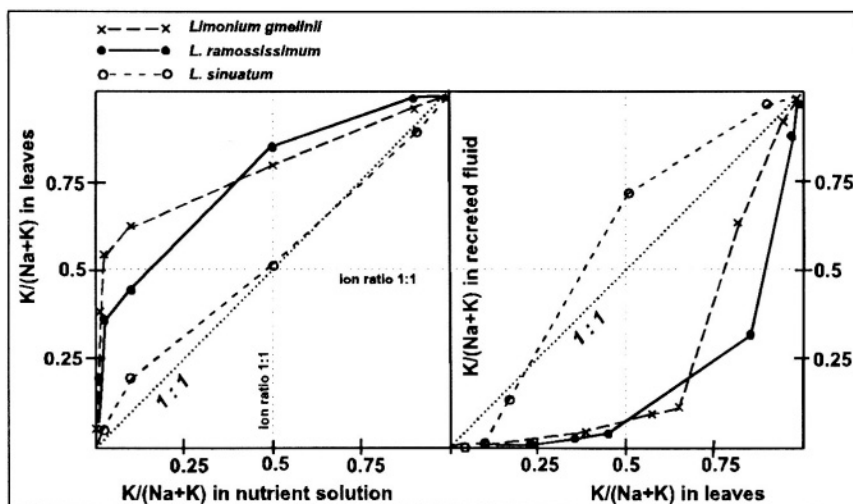


Figure 6. Ion ratios (K/Na+K) in three *Limonium* species differing in salt tolerance. Left side: Correlation between nutrient solution and leaves; right: Correlation between leaves and recreted fluid from salt glands (Wiehe, 1986)

apoplastic backflow of salt from the bladder to the mesophyll (Freitas and Breckle 1993).

Accumulation of sodium and chloride in the bladders was detected in many species of *Atriplex*, suggesting their role in the ionic balance of these plants (Freitas and Breckle 1994). According to Osmond (1979), more than 50% of chloride content in *Atriplex* leaves is recreted to the bladders. The percentage of sodium and chloride in bladders in relation to leaves in *Atriplex rosea* was rather similar in different salt treatments (Freitas and Breckle, 1994), no increment was verified in the highest treatments. The contribution of bladder hairs to the regulation of sodium and chloride is, to some extent, determined by the salt concentration of the leaves. Even excessive nitrate can be recreted to bladders. In young leaves of seedlings the ionic balance and regulation ability is not well developed. Thus, available ions are accumulating in the leaves, and bladder hairs become an important mechanism for ionic balance for the developing leaves (Freitas and Breckle, 1994).

Similar bladders as in *Atriplex* are reported for *Chenopodium*, *Salsola*, *Halimione* etc. The ecological significance of bladder bearing species is still under dispute, but if one takes into account, that vast areas of semideserts are covered by low shrubby vegetation of *Atriplex* and other chenopods, it can be suggested, that this is likely to be one of the successful mechanisms for adaptations to salinity.

Conversely, the huge epidermal cells of the Mesembryanthemaceae (Aizoaceae) do not appear to function as salt deposition structures, and function probably as water storage tissues (see Chapter 16).

3.3.6 PSEUDOHALOPHYTES AND NON-HALOPHYTES

Saline vegetation types often are composed of several species, where some of these species are not real halophytes. They occur only accidentally in such plant-communities of oligohaline marshes, but have their optimal growth and performance in nonsaline vegetation. These plants might have a slightly higher salt tolerance (Howard and Mendelssohn, 1999). Normally such plants lack specific anatomical features, but may become slightly succulent under salinity as a modificative character (*Plantago major*). Non-halophytes (Glycophytes) also can become somewhat succulent under salt stress, as was demonstrated with many cultivated species (Stroganov, 1964). The strong formative effects on leaf structure of saline conditions were shown by Waisel (1972). Not only does salinity increase the cell size, but also it changes the density of stomates, changes the ratio of palisade and spongy parenchyma etc.

The number of species which exhibit special salt tolerant ecotypes is rather large. Ellenberg *et al.* (1991) have characterized them by an indicator value for halophytic performance (see Table 4).

3.3.7 HALOPHILIC GRASSES

There are several grasses which are highly salt-tolerant (Table 4); for C4-grasses see Chapter 16, but euhalophytic halo-succulents, are not represented among the grasses.

Although osmotic adjustment by storage of organic solutes is regarded more costly energetically than storage of Na^+ and Cl^- (Yeo, 1983), in salt-excluding species the more salt-resistant ecotypes are those with a greater restriction of Na^+ -**accumulation** in the shoots. Comparing two populations of *Hordeum jubatum* from a saline and one of a nonsaline site, Wang *et al.* (1992) found lower Na^+ -**concentrations** in the shoots of the plants originating from the saline habitats, when they were treated with 148 mol m^{-3} Na^+ in hydroponic solutions. This confirms findings by Rozema (1976) on ecotypes of *Juncus*, it seems to be rather characteristic for graminoids, which, as a rule, are very efficient salt-excluders (Albert, 1982). In the salt-secreting grass *Spartina alterniflora* a dwarf ecotype was recently found (Daehler *et al.*, 1999).

They use 10 numerals for characterizing ecological behaviour of a plant species in relation to salinity. On the other hand, the occurrence of those species can be used to

3.4 Natural saline ecosystems

3.4.1 SALINE COASTAL ECOSYSTEMS

There are many descriptive accounts on salt marsh vegetation (e.g. Adam, 1990). The main approaches are using structural and physiognomic attributes and/or floristic data for circumscription of plant communities. The initial similarity in appearance of salt marshes allows only a small number of categories, hence salt marshes are also treated as azonal vegetation types. On the basis of their dominant growth form it is possible to recognize three categories:

- Herb communities
- Graminoid communities (with grasses, sedges, and rushes)
- Dwarf shrub communities

At most sites all three types of communities co-exist. However, arctic and boreal marshes are predominantly covered by graminoid marshes, while dwarf shrub marshes are extensively developed along arid and semi-arid tropical and subtropical coasts.

The lifeforms of halophytic species in salt marshes are predominantly Hemicryptophytes (acc. to Waisel, 1972, e.g., in Europe 40%; in Israel 35%), Therophytes (30%; 19%), Chamaephytes (10%; 29%); Phanerophytes (0%; 17%) and Cryptophytes (10%; 0%). In most salt marshes all over the world the hemicryptophytes are the dominant lifeform, only in the tropics (mangrove) the phanerophytes dominate. The question, why there are no salt tolerant bigger shrubs and trees in the temperate climatic zones is still an open question (Tomlinson, 1986) and selection of trees for high salinity tolerance is a big need (Sun and Dickinson, 1995).

Coastal salt marshes and mangroves are found between the high tide and the near-shore sublittoral zones, not only along coasts, but also in estuaries of continents. They often flourish in regions where much silt is carried to the coastal regions by rivers or where geological processes favor erosion and suspension of silt. Salt marshes have a high rate of primary productivity. Processes in salt marshes are governed by the interactions of abiotic (physical, chemical and geological) and biotic (herbivory, parasites, decomposition) factors (Vernberg, 1993). Above-ground vegetation and seed bank composition of zonal communities along salinity gradients is usually not highly correlated. Since seeds of annual salt marsh species occur in all zones, the levels of salt stress may be the main factor determining which species is found in the above-ground vegetation (Egan and Ungar, 2000). Material cycling, biogeochemical and nutrient cycling as well as overall productivity for ecosystem functions and interactions with adjacent ecosystems are major processes and are suspect to typical long-term changes, since salt marshes are typical ecotones.

3.4.1.1 *Salt marshes in temperate regions*

By using the dominant species concept a number of salt marsh communities can be distinguished. In addition, however, the full floristic composition gives a better picture

in species-rich communities on the recurrent group of species. Normally salt marshes are poor in species and individual species in most genera have a wide ecological amplitude, except e.g. in *Limonium*.

Along the zonation with vegetation belts parallel to the sea shore, with increasing elevation and decreasing number of tidal inundations these vegetation belts exhibit a strong ecological gradient. Salinity decreases with elevation. The salinity limits between the vegetation belts predominantly determine the occurrence of the phytosociological vegetation units (Figure 7) by competition between species as members of the units.

Salinity normally is negatively correlated with species richness (Garcia *et al.*, 1993). A comparison of zonation pattern between larger areas is difficult because the tidal influence (number and duration of submergences) may be different as well as the floristic pattern, the geographical and climatical features and substrate conditions.

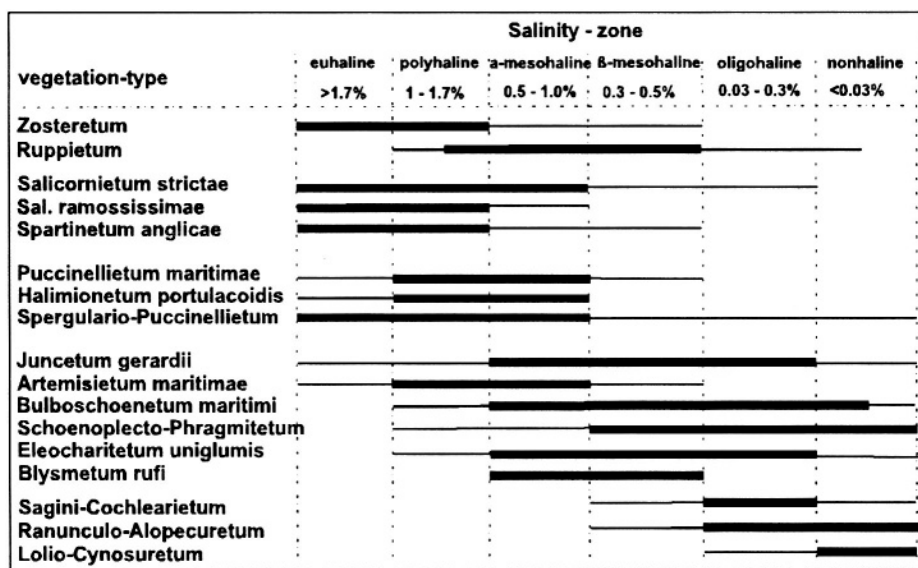


Figure 7. Optimal ranges and amplitudes in salinity of main vegetation types along Southern Scandinavian coasts (Dierssen *et al.*, 1991; Dierssen, 1996)

Substrate conditions according to sedimentation may be altered by trapping of sediments. This may lead to a long term succession with longtime stable vegetation limits, but by sudden episodic events, such as storms or strong floods the present vegetation mosaic might undergo rapid changes in species composition.

With increasing elevation, species diversity and diversity of plant communities tends to increase. The extreme upper marsh, the ecotone to the non saline plant communities, is the transition to other habitats. It is a region of high heterogeneity, strong

environmental gradients and considerable temporal variability. This narrow zone normally supports a wide range of communities and a rich flora.

3.4.1.2 *Mangrove forests*

Mangroves (see Chapter 6) occupy the tropical and subtropical habitat equivalents of the salt marshes of the temperate regions. In arid regions the mangrove is separated from inland vegetation by an open extremely saline flat, often bare of vegetation, whereas in tropical humid areas the salinity gradually decreases (Walter and Breckle, 1986, 1991).

3.4.2 SALINE INLAND ECOSYSTEMS

3.4.2.1 *Geogenic saline vegetation in humid climates*

In humid regions inland salines are sparse. They exist only on geogenic features, where salt-springs come to the surface. Leaching by rain leads to a steady reduction of salinity unless salt is replaced from geological sources. Those inland salines are normally rather small in area, but nevertheless have a rich flora in Central Europe (Wendelberger, 1950). Soil chemistry might differ from one place to another in contrast to coastal marshes, where the similarity in ion chemistry is governed by the overall influence of sea-water.

3.4.2.2 *Salt deserts in arid climates*

In deserts xerohalophytes are often dominant plant species as a result of coping with the accumulation of salt in soil during long times. In many deserts the mosaic of ecotopes corresponds to a topo-sequence from mountains to accumulation basins. Along these oblique peneplains the particle size in the soil decreases, from rocks, gravel, sand to silt and clay material, giving rise to different desert types, and according to the relevant abiotic transporting means water and wind, sand or loess deserts develop. In the lowest parts the finest particles especially clay and soluble salts accumulate, forming salt lakes with salt deserts.

Within the various desert types saline soils are unevenly present, since geochemic transportation of soluble salts normally results in accumulation in erosion basins. The ecogram of dominant chenopod-halophytes in Northeastern Iran and in the West- and North-Afghanistan (Figure 8), where a highly diverse halophytic flora has evolved, exhibits members in each niche (Breckle, 1982).

The sequence of species along the salt gradient in a rich halophytic area, as it is in the Central Asian deserts, reveals a typical sequence of the dominating halophyte types (Figure 9). Along the salt gradient (Breckle, 1986), which can be derived from salinity measurements in a mosaic vegetation, too, it is obvious, that the stem-succulents (S) and then the leaf-succulents (L) play the major role close to the saline lakes or basins. The recreting halophytes (X) dominate in the middle part of the transect. This part of the transect is often also characterized by less water availability and often here a much higher proportion of C4-plants occur. This is also the case on the less saline side, where then pseudohalophytes (P) and finally on almost saltfree substrates the

nonhalophytes (N) predominate and other ecological factors, such as, e.g., water-availability and -supply, nitrogen-source etc. are governing the vegetation mosaic.

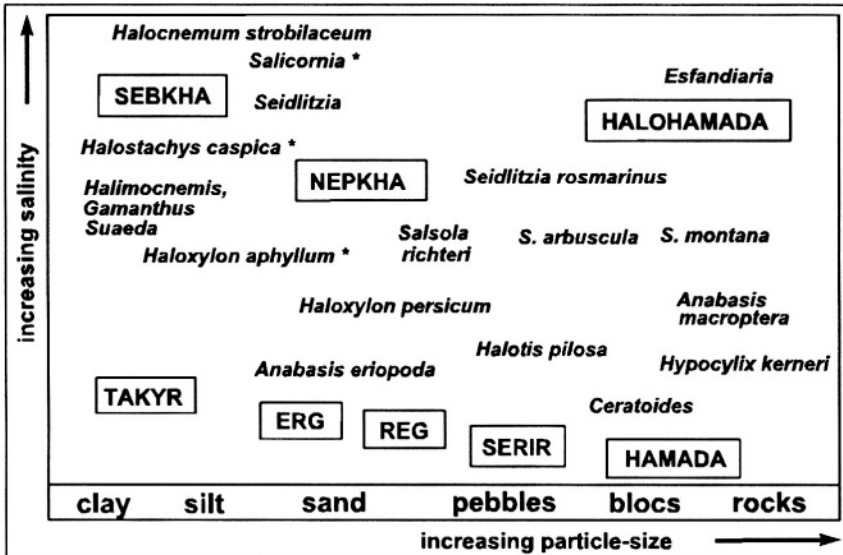


Figure 8. Ecogram of Chenopodiaceae species and correlation to various desert types (mainly for Northeastern Iran and North-Afghanistan). [* = with additional water supply] (Breckle, 1982). Hamada (rock desert), Serir (pebble desert), Reg (sand and stone pavement desert), Erg (Sand dune desert), Takyr (clay plains) are geomorphological desert types according to substrate particle size; Sebkhah (salt desert, salt pans), Nephkah (small, mostly salty sand dunes) and Halohamada (salty bloc desert) are geomorphological desert types under influence of salinity)

Halo-Type		← % NaCl in soil												
		15	10	5	3	2	1	0.5	0.2	0.1	0.05	0.02	0.01	<0.01
S		71	63	51	18	3	12	7	4	11	10	•	•	
L		29	32	30	72	30	56	51	18	35	18	10	•	
X		•	5	19	•	60	27	28	41	4	9	•	•	
P		•	•	•	5	7	5	15	23	24	45	68	10	
N		•	•	•	•	•	•	•	14	26	24	22	88	

Figure 9: Abundance of halophyte types (% of total species) in halo-catenas in Iran along the salinity gradient (soil salinity, % of dry matter; explanation for S-L-X-P-N, see Figure 1) (Breckle, 1986)

An almost similar sequence of halophyte-types can be derived from the vegetation belts around Great Salt Lake in Utah (Kearney *et al.*, 1914). The ecogram (Figure 10) reveals the similar sequence of S-L-X-P-N halophytes along the salt gradient.

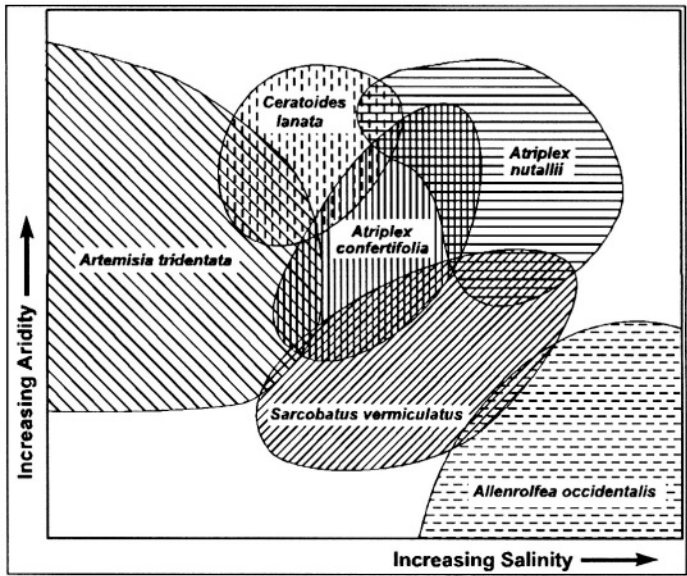


Figure 10. Ecogram showing range of dominant species derived from transects of the Great Salt Lake salt desert according to salinity and aridity (water availability) (Breckle, 1976)

Halophytes often change their substrate themselves by their accumulation of salts and absorption of salts from the soil. This causes on the long run a typical heterogenic salt pattern, with a decreased salinity under the shrubs (Figure 11). The litter, rich in salt, is blown away from the shrubs. In other halophytic communities, however, it could be demonstrated, that saltrich litter is accumulating under the shrubs, such as, e.g., under *Anabasis articulata* in the sanddune ecosystems of the Negev-desert at Nizzana (Veste and Breckle, 1995). Interrelations of plants and soil thus can be very different, according to prevailing factors.

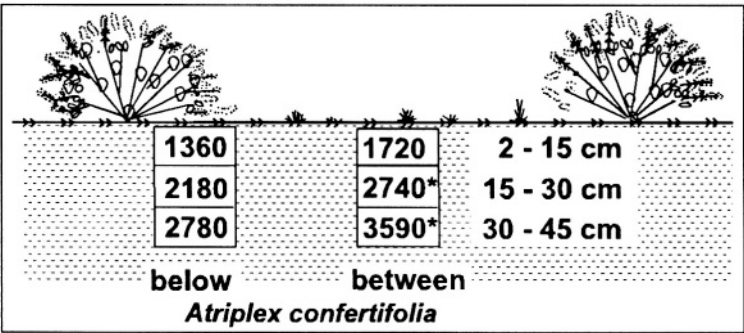


Figure 11. Mosaic of salt concentration in soil shown by significantly different Cl⁻-values in ppm per dry matter between and below halophytic shrubs of *Atriplex confertifolia* (Curlew Valley, Northern Utah, Breckle, 1976).[* : significantly higher values at the 1% level, n = 10]

3.5 Conclusions and Outlook

Adaptive responses among various species show that salt tolerant plants have evolved specialized complex mechanisms, which allow adaptation to saline stress conditions. On the level of tissues, such unique structures as, e.g. salt glands, exist in some plant taxa. Cellular based mechanisms appear to be common in many genotypes and are a prerequisite for salt tolerance, especially the regulation of osmotic adjustment by uptake of inorganic ions (Chapter 8) and/or by synthesis of compatible solutes (Chapter 9). The accumulation and intracellular compartmentation of ions is mainly the result of active transport mechanisms across the plasma membrane and the tonoplast (Chapters 18, 19). Thus all complexity levels have to be taken into account, when considering salinity effects on plants or even ecosystems.

In the future it may turn out that many genes are involved in the complex salt tolerance syndrome (Chapters 21 and 22).

It might be important to find ways, e.g., by quantitative modelling, to extrapolate from one to the next system of complexity. The uppermost level of complexity: the saline ecosystems, salt marshes and mangroves, need special attention, since they are often sensitive biological areas. The intertidal ecosystems are often very narrow and fragile strips, in many countries already almost totally destroyed but with a very high ecological and potential economical importance.

Ecology of halophytes is still a challenging subject. In spite of research on halophytes for more than 100 years even some basic questions are still unanswered, but will be important, e.g., for breeding halotolerant crops from used crop varieties or by breeding new crops from natural halophyte species. Ion effects, nutrient conditions, osmotic effects, growth and carbon partitioning, intracellular compartmentation, cell wall adjustment and many other interrelated processes on the autecological level have to be checked further in the future. Ecotypes and biogeography, germination and establishment, competition and nutrient availability under high salinity and alkalinity are subjects on the ecosystems level which have to be investigated further.

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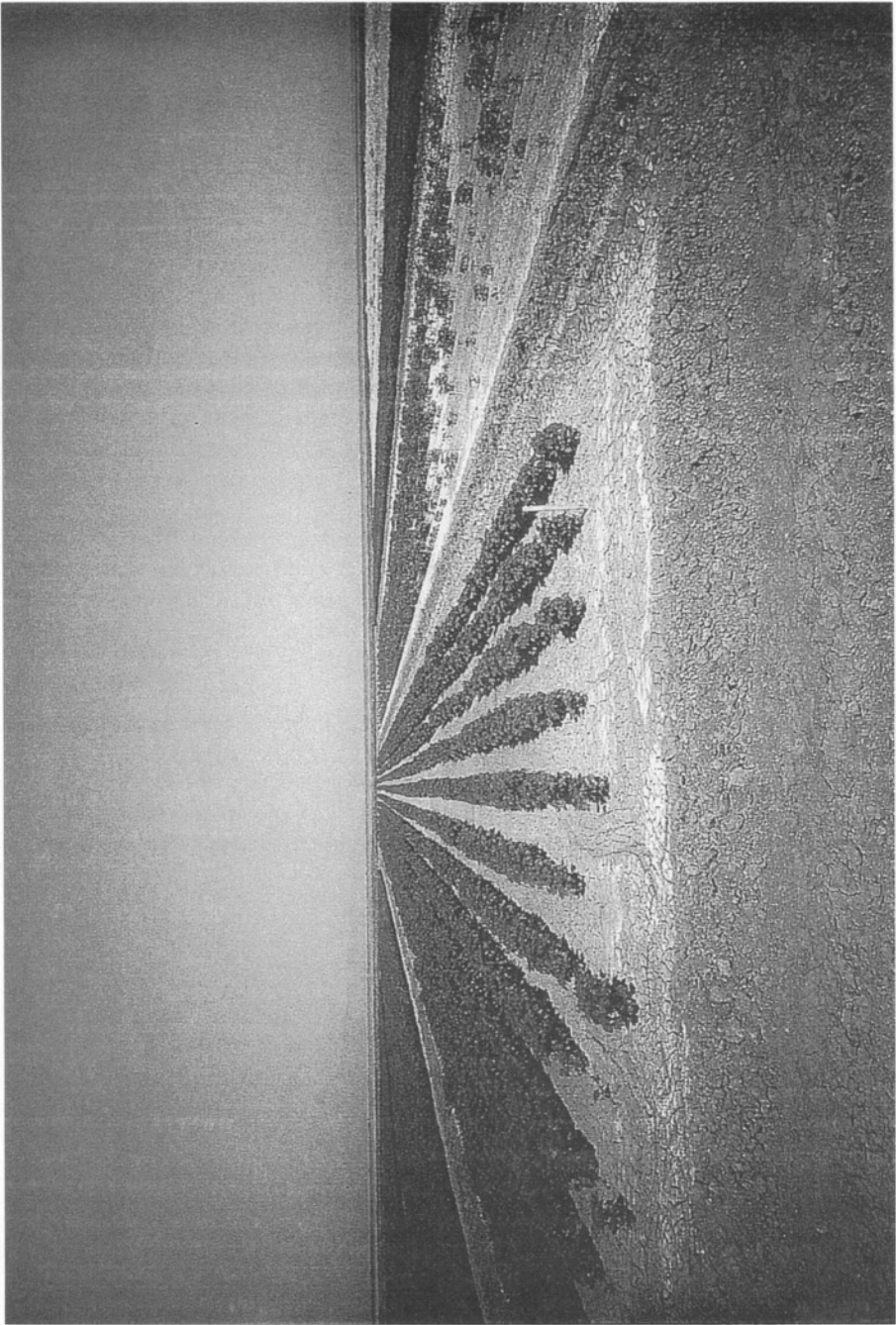
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B. ORGANISMS

While, in this book, we are mainly interested in higher plants - as highlighted by our picture of a crop of cotton plants in a field after irrigation with saline drainage water (salinity of irrigation water: control on the upper left, 4500 ppm total dissolved salts in the middle, 9000 ppm on the right of the picture; third year of saline water irrigation; San Joaquin Valley, California, June 1987) - it appears essential to look at organisms at different levels of organization. Prokaryotes and single celled eukaryotes can make up a substantial fraction of the biomass in some ecosystems. Moreover, they can tell us much about possible basic mechanisms for coping with high salinities. It has even been argued quite frequently that only among prokaryotes there are real *bona fide* halophilic organisms that are obligatorily restricted to high salinity for their growth. On the other hand, halophytes among plants, although often tolerant of high salinity, mostly appear facultative with respect to their salt requirements. Thus, comparisons at different levels of organization of organisms may also give us hints on evolutionary aspects of halophily *sensu stricto* and mere salt tolerance, respectively.

Among the prokaryotes halophilic taxa are concentrated in the domain of the Archaea (Chapter 4). Moving on to the case study of the halophilic unicellular alga *Dunaliella* then introduces compartmentation as a new feature of eukaryotic cells dealing with salinity stress (Chapter 5). Further below (Chapter 21) we shall draw on an unicellular eukaryotic organism again, namely yeast, to view genes of halotolerance.

Division of labor between organs of higher plants - roots, stems, leaves, flowers and fruits - then further increases the degree of complexity and the number of levels, where responses to salinity may occur. There is a large diversity of life forms of higher plants adapted to salinity, which has already been described in Chapter 3 above as a basis of delineating salt affected natural ecosystems. In addition mangroves and mangrove plants are a very conspicuous ecosystem and life form (Chapter 6).



CHAPTER 4

ADAPTATION OF HALOPHILIC ARCHAEA TO LIFE AT HIGH SALT CONCENTRATIONS

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Abstract

The halophilic Archaea (order Halobacteriales) form a diverse group of microorganisms adapted to life at high salt concentrations. The cells contain molar concentrations of K^+ and Cl^- to provide osmotic balance. Enzymes and other proteins require the presence of high salt concentrations for activity and structural stability. Most proteins contain a large excess of acidic amino acids and low amounts of hydrophobic amino acids. The structure resolution of a number of such enzymes by X-ray crystallography and the use of site-directed mutagenesis in recent years has greatly increased our understanding of the adaptation of the Halobacteriales to life at high salt concentrations.

4.1 Introduction

Many microorganisms, prokaryotic as well as eukaryotic, have learned to cope with the presence of high salt concentrations in their environment. Indeed, salt lakes with salt concentrations approaching saturation, such as the Dead Sea and the Great Salt Lake (Utah), and saltern evaporation ponds for the production of solar salt, may be inhabited by dense communities of halophilic and halotolerant microorganisms.

As all biological membranes are highly permeable to water, any microorganism living at high salt has to maintain its intracellular environment at least isoosmotic with the salt concentration in its environment. In order to achieve the required osmotic balance, nature has devised two fundamentally different strategies for halophilic and halotolerant microbes enabling them to cope with the osmotic stress exerted by the high ionic strength of their medium. The first strategy, used by halophilic and halotolerant algae and by all known halotolerant aerobic representatives of the domain Bacteria, is to avoid high salt concentrations from reaching the cytoplasm. Their enzymes are “con-

ventional” proteins, not specifically designed to function at high ionic strength. The presence of high salt concentrations will cause them to become inactivated and/or to aggregate by “salting-out” (see section 4.4.2), and as a result the whole intracellular machinery will cease to function. Low intracellular ionic concentrations are maintained by active pumping of ions out of the cells and by the production of organic osmotic solutes or accumulation from the medium. The unicellular green alga *Dunaliella* uses glycerol for this purpose (Chapter 5), and a wide variety of organic “compatible” solutes function in the aerobic halophilic Bacteria (Ventosa *et al.*, 1998). The use of organic osmotic solutes bestows a high degree of flexibility to these microorganisms. The intracellular solute concentrations are regulated in accordance to the external salt concentration, and they can be rapidly adjusted when the salinity changes.

The aerobic halophilic Archaea (family *Halobacteriaceae*) use a different strategy to cope with the high salt concentrations (generally above 20% salt, and up to NaCl saturation) in which they thrive. Their cytoplasm contains molar concentrations of salts, mostly KCl, providing the necessary osmotic equilibrium. The presence of high internal salt concentrations makes it essential that the whole metabolic and regulatory cell machinery be extremely halotolerant. The presence of K^+ , Cl^- , and other ions in molar concentrations requires a far-going adaptation of the intracellular enzymes, which have to be active in this high-ionic-strength medium. As discussed below, the adaptations of the proteins are such that they are not only able to function in the presence of high salt concentrations, but they also show an absolute requirement for molar concentrations of salt to maintain activity and stability. As a result the aerobic halophilic Archaea display much less flexibility and adaptability to changes in the external salt concentration. Most of their proteins, including the cell wall glycoprotein that gives mechanical strength to most species, require the continuous presence of high salt, and they will denature when suspended in solutions containing less than 1-2 M salt. This strategy of “salt-in” to balance “salt-out”, with adaptation of the cellular components to function in the presence of high ionic concentrations, is in addition found in a small group of obligatory anaerobic halophilic Bacteria (families *Haloanaerobiaceae* and *Halobacteroidaceae*) (Oren, 1986; Oren *et al.*, 1997). These are less halophilic than the aerobic Archaea of the family *Halobacteriaceae*, and much less is known on their haloadaptation at the molecular level.

Below I will present some of the unique features of the halophilic Archaea, with special emphasis on the way these fascinating microorganisms are adapted to function in the presence of near-saturating salt concentrations.

4.2 The aerobic halophilic Archaea - a diverse group

In a classic essay on “the halobacteria’s confusion to biology”, Helge Larsen exposed some of the unique features that distinguish the halophilic Archaea of the family *Halobacteriaceae* from all other forms of life (Larsen, 1973). These organisms are found worldwide in almost any environment in which NaCl concentrations approach saturation. They may impart a reddish-purple tinge to the water of the Great Salt Lake and to the Dead Sea, they color the brines in saltern crystallizer ponds brightly red, and they

are also often found as red colonies on salted fish and on hides treated with salt for preservation (Bayley and Morton, 1978; Kushner, 1985; Oren, 1994).

Our understanding of the diversity within this group of Archaea has greatly increased during the last two decades. In addition to the long known rod-shaped *Halobacterium salinarum*¹ and the coccoid *Halococcus morrhuae*, many additional types have been isolated. Some of these require lower salt concentrations, and may be considered moderate rather than extreme halophiles (e.g. *Haloferax mediterranei* and *Haloferax volcanii*). Some show only slight pigmentation (*Haloferax mediterranei*) or may be altogether colorless (*Natrialba asiatica*). Some have a high tolerance and requirement for divalent cations such as Mg^{2+} (e.g. *Halorubrum sodomense*) (Oren, 1983), and alkaliphilic species such as *Natronobacterium gregoryi* and *Natronococcus occultus* combine a high salt requirement with a requirement for high pH values for growth (optimum pH 9-10) and a low requirement for divalent cations. An overview of the properties of these and other halophilic Archaea was given by Tindall (1992).

The basic metabolism of all members of the *Halobacteriaceae* is chemoorganotrophic, aerobic. They respire a variety of organic compounds, including amino acids and to a lesser extent carbohydrates, using oxygen as terminal electron acceptor. The group displays a considerable metabolic diversity. Several members (e.g. *Haloarcula marismortui*, *Haloferax denitrificans*) are able to grow anaerobically using nitrate as terminal electron acceptor (Mancinelli and Hochstein, 1986). *Halobacterium salinarum* can grow anaerobically by fermenting arginine (Hartmann *et al.*, 1980), an ability that appears to be absent in all other non-alkaliphilic species tested. *Halobacterium salinarum* is also capable of growing anaerobically using light as energy source when the cells have earlier been induced to produce the light-driven proton pump bacteriorhodopsin (Hartmann *et al.*, 1980). The ability to use light energy may enable certain members of the *Halobacteriaceae* to survive prolonged periods of starvation for suitable organic carbon sources (Oren, 1994).

The discovery of the retinal proteins bacteriorhodopsin (an outward proton pump) and halorhodopsin (an inward chloride pump) contributed much to the interest of the scientific community in the halophilic Archaea. These proteins became well-investigated models for energy conversion, leading to the conservation of light energy into forms directly usable by the cells. An in-depth discussion of these proteins and their photocycles is beyond the scope of this chapter, as these proteins do not require any salt for activity and stability, and as such they are highly unusual among the proteins of the halophilic Archaea. Additional details can be found in review papers on the subject (Lanyi, 1986, 1990; Lanyi and Váró, 1995; Oesterhelt, 1995).

In spite of their limited adaptability to variations in salt concentration, the halophilic Archaea form a highly successful group of microorganisms, which encounter little or

¹ In accordance with the presently accepted nomenclature of halophilic Archaea species, strains previously designated as *Halobacterium halobium*, *Halobacterium salinarum*, and *Halobacterium cutirubrum* are now named *Halobacterium salinarum* (Ventosa and Oren, 1996). Many other species have recently been assigned to new genera, and thus the nomenclature used in this chapter may differ from the names used in the original papers cited.

no competition from other bacteria in hypersaline environments of sufficiently high salt concentrations (Oren, 1994). They may survive long periods of starvation by mechanisms not yet understood. Viable halophilic archaeal cells have even been recovered from Triassic and Permian British salt mines (Norton *et al.*, 1993). However, a critical discussion of the experimental evidence showed only little evidence for longevity of halophilic Archaea in subterranean salt deposits (Grant *et al.*, 1998).

4.3 Intracellular ionic concentrations of halophilic Archaea

4.3.1 IONIC CONCENTRATION MEASUREMENTS

Since the first attempts to measure concentrations of intracellular ions in a *Halobacterium* and a *Halococcus* strain by Christian and Waltho (1962) it is clear that the aerobic halophilic Archaea contain molar concentrations of ions, especially K^+ and Cl^- , with often considerable concentrations of Na^+ being reported as well. Table 1 summarizes some estimates of intracellular ionic concentrations in different representatives of the halophilic Archaea.

Most estimates of intracellular ionic contents are based on the analyses of pellets of packed cells following centrifugation. Distribution of different non-penetrating solutes, such as phosphate, inulin, and others, has been used by different workers to correct for the volume of the intercellular space within these pellets. The values reported by different authors vary considerably, and they also depend on the species tested and the physiological state of the cells (exponential vs. stationary growth phase). However, it is evident in every case that the apparent total intracellular concentration of monovalent cations exceeds their external concentration. In those cases in which intracellular chloride concentrations were measured as well, Cl^- was also found inside the cells in molar concentrations.

Another approach used to measure intracellular ionic concentrations, this time in single cells, is X-ray microanalysis with the transmission electron microscope (Oren *et al.*, 1997). *Halobacterium salinarum* cells grown in medium containing 4.3 M NaCl had apparent intracellular K^+ , Na^+ and Cl^- concentrations of 1.95, 0.35 and 1.88 M, respectively, being about half of the values expected. This discrepancy may be due to artifacts caused by the air-drying involved, which may result in a flattening of the cells, leading to an overestimation of the true dimensions and volume of the cell.

4.3.2 ION PUMPS AND OTHER MECHANISMS INVOLVED IN THE REGULATION OF INTRACELLULAR IONIC CONCENTRATIONS

Analyses of intracellular ionic concentrations in different halophilic Archaea, as presented in Table 1, not only show that these microorganisms maintain extremely high salt concentrations inside their cells, but also that the ionic composition of their intracellular milieu differs greatly from that of their outside medium. Notably the intracellular concentrations of potassium are extremely high, and it was claimed that K^+ ions

can represent up to 30-40 percent of the cell dry weight. The huge potassium concentration gradient (up to three orders of magnitude) over the cytoplasmic membrane, and also the often large sodium gradient present, can only be created and maintained at the expense of large amounts of energy. Also the chloride ion is far from equilibrium, as the presence of a membrane potential (inside negative) would tend to expel Cl^- from the cell. The peculiar ionic composition of the cells' cytoplasm and the concentration gradients involved are the result of the cooperative action of a number of bioenergetic processes and ion pumps, and these are summarized in Figure 1.

Being chemoorganotrophic aerobic microorganisms, the primary bioenergetic process enabling the cell to obtain energy to drive endergonic reactions is respiratory electron transport, using oxygen as the terminal electron acceptor. As in other similar processes in non-halophilic bacteria, this electron transport is accompanied by the extrusion of protons, generating a primary proton electrochemical gradient (acidic outside, alkaline inside, positive outside, negative inside). This proton gradient is then directly or indirectly the driving force for all energy requiring processes within the cell. One of these processes is the formation of ATP from ADP and inorganic phosphate, mediated by the membrane-bound ATP synthase which drives phosphorylation of ADP coupled with an inward flux of H^+ . Another energy-requiring process driven by the proton gradient is flagellar motion, present in many (but not all) halophilic Archaea.

Alternative modes of generation of the proton electrochemical gradient exist in the halophilic Archaea: as stated above, some species may replace oxygen as the terminal

TABLE 1. Estimates of intracellular ionic concentrations in aerobic halophilic Archaea of the family *Halobacteriaceae*.

Species	Medium concentration				Intracellular concentration				Reference
	Na^+	K^+	Mg^{2+}	Cl^-	Na^+	K^+	Mg^{2+}	Cl^-	
<i>Halobacterium salinarum</i>	4.0	0.032			1.37	4.57		3.61	Christian and Waltho, 1962
<i>Halobacterium salinarum</i> ^b	3.7	0.013	0.1		1.63	2.94			Lanyi and Silberman, 1972
<i>Halobacterium salinarum</i> ^b	3.33	0.05	0.13		0.80	5.32	0.12		Matheson <i>et al.</i> , 1976
<i>Halobacterium salinarum</i> ^d	4.3	0.067	0.025	4.5	0.35	1.95		1.88	Oren <i>et al.</i> , 1997
<i>Haloarcula marismortui</i> ^a	3.9	0.004-0.007	0.15	3.9	1.2-3.0	3.77-5.5		2.3-4.2	Ginzburg <i>et al.</i> , 1970
<i>Haloarcula marismortui</i> ^b	3.9	0.001-0.004	0.15	3.9	1.6-2.1	3.7-4.0		3.2-4.1	Ginzburg <i>et al.</i> , 1970
<i>Haloarcula marismortui</i> ^c	3.9	0.0075	0.15	3.9	0.5-0.7	3.7-4.0		2.3-2.9	Ginzburg <i>et al.</i> , 1970
<i>Halococcus morrhuae</i>	4.0	0.032			3.17	2.03		3.66	Christian and Waltho, 1962

Concentrations are in molar units, except those relating to *Haloarcula marismortui*, which are in molal units. ^a Early exponential growth phase cells; ^b Late exponential growth phase cells; ^c Stationary growth phase cells. ^d Data obtained by X-ray microanalysis in the electron microscope. Values of intracellular concentrations may underestimate the true values. For additional information see text.

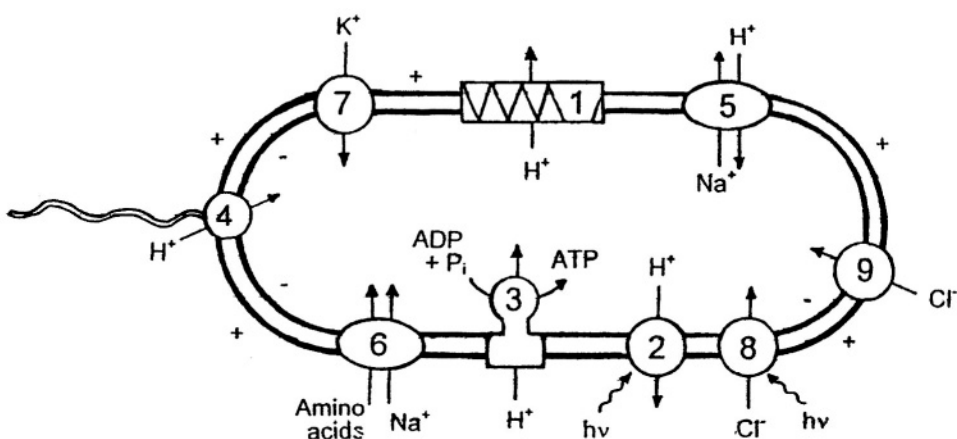


Figure 1. Ion movements in the aerobic halophilic Archaea (family *Halobacteriaceae*). 1, proton extrusion via respiratory electron transport; 2, light-driven proton extrusion mediated by bacteriorhodopsin; 3, ATP formation by ATP synthase, driven by the proton gradient. Alternatively this system can serve to generate a proton gradient at the expense of ATP during fermentative growth on arginine; 4, proton-gradient driven flagellar motor; 5, electrogenic sodium/proton antiporter; 6, sodium gradient-driven inward amino acids transport; 7, potassium uniport, driven by the membrane potential; 8, primary, light-driven chloride pump, mediated by halorhodopsin; 9, light-independent chloride transport system, possibly coupled with inward transport of sodium. For details see text.

electron acceptor with other oxidized compounds, notably nitrate. Those species that contain the retinal protein bacteriorhodopsin may use light energy for the direct generation of a proton electrochemical gradient. Finally, *Halobacterium salinarum* growing anaerobically on arginine obtains its energy from substrate-level phosphorylation in the form of ATP. The membrane-bound ATP synthase then acts as an ATPase, transporting protons from the cytoplasm to the outer medium accompanied by ATP hydrolysis.

The proton electrochemical gradient is the driving force for the extrusion of Na^+ from the cell, keeping intracellular Na^+ concentrations relatively low. Membranes of halophilic Archaea possess a very high activity of an electrogenic Na^+/H^+ antiporter. This was demonstrated in experiments in which bacteriorhodopsin-containing right-side-in membrane vesicles of *Halobacterium salinarum* were illuminated: following the formation of the primary light-driven proton gradient, Na^+ was found to be extruded from the vesicles at the expense of the proton gradient. The antiporter is electrogenic, and probably has a stoichiometry of $2\text{H}^+/\text{Na}^+$ (Lanyi and Silverman, 1979). Also artificial electron donors will drive a rapid Na^+ extrusion from the vesicles in the dark, showing

that the antiporter activity is independent of the presence of photoactive pigments (Lanyi and MacDonald, 1976; Luisi *et al.*, 1980).

The sodium gradient thus established can in its turn be used to drive certain endergonic processes. The existence of **Na⁺-driven** cotransport of amino acids through the membranes of halophilic Archaea is well established. It has also been suggested that the light-independent inward transport of chloride (see below) may be driven by the sodium gradient, using a **Na⁺/Cl⁻** symport system (Duschl and Wagner, 1986).

It is generally accepted that the membrane potential [estimated to be in range of 100–150 mV, negative inside (Bayley and Morton, 1978)] is the driving force for the massive **K⁺** accumulation. The membranes of halophilic Archaea were found to be highly permeable to potassium. **K⁺** ions probably enter the cells via a uniport system in response to the membrane potential (Wagner *et al.*, 1978). **K⁺** enters the cell as **Na⁺** is ejected by the electrogenic **Na⁺/H⁺** antiporter, thus maintaining electroneutrality. Evidence for such a mechanism was found in experiments showing accumulation of radioactively labeled rubidium (a potassium analog) ions in right-side-out vesicles of *Halo-bacterium salinarum* as a reaction to sodium extrusion following excitation of the bacteriorhodopsin light-driven primary proton pump (Kanner and Racker, 1975). In addition, a **K⁺** transport system analogous to the Kdp system of *Escherichia coli* was detected in *Haloferax volcanii* (Meury and Kohiyama, 1989). This system requires ATP for activation.

Due to the presence of the membrane potential (negative inside), the intracellular chloride concentrations measured in different types of halophilic Archaea are much above the values expected on the basis of an electrochemical equilibrium. It is clear that the high internal **Cl⁻** concentration is not in equilibrium with the large negative-inside electrical potential that accompanies the **H⁺** circulation and the **Na⁺** efflux. Thus, electrical potential-driven passive chloride movement can result only in chloride loss from the cells rather than in the required uptake. For these reasons it is necessary to postulate active inward chloride transport in these cells. If the cells should increase their volume during growth and cell division, an increase in the amount of intracellular **Cl⁻** is essential. It has been suggested that during growth the net flux of ions should result in **K⁺** uptake in excess of **Na⁺** loss, and a **Cl⁻** uptake should be equal to the difference, so as to provide net gain of intracellular KCl commensurate with the gain in intracellular volume (Lanyi, 1986; Schobert and Lanyi, 1982).

At least two energy-dependent inward chloride pumps have been identified. One is halorhodopsin, a light-dependent primary inward **Cl⁻** pump, present in *Halobacterium salinarum*, in *Natronomonas pharaonis*, and probably in additional halophilic Archaea as well (Oesterhelt, 1995). As all halophilic Archaea are able to grow in the dark, light independent **Cl⁻** transport systems should also be present. A highly efficient secondary chloride uptake system, energized by respiration or by light via bacteriorhodopsin, and independent of halorhodopsin, has been described. Its activity was not correlated with the size of the proton gradient, and **Cl⁻** influx under respiratory conditions may even be increased with a decreasing proton gradient. This inward **Cl⁻** transport is probably linked to the membrane potential, and was suggested to be driven by symport with **Na⁺** (Duschl and Wagner, 1986).

4.3.3 THE STATE OF BINDING OF POTASSIUM IONS IN THE CYTOPLASM

The presence of apparent KCl concentrations as high as 5 M in the cytoplasm of halophilic Archaea, a concentration well beyond the solubility of KCl in water, raises the question whether the potassium ions occur freely dissolved or may be bound in massive amounts to proteins and other intracellular structures (Kushner, 1988). Some indications have been found that part of the K^+ may be tightly bound within the cells. Thus, high apparent intracellular K^+ concentrations may be maintained for long periods also in the absence of an energy source, and even in poisoned cells. Unless K^+ is specifically bound onto intracellular molecules it is difficult to understand how large amounts of the ion can be retained. Ginzburg (1978) and Ginzburg *et al.* (1970) suggested that potassium ions may indeed be tightly bound inside the cytoplasm of *Haloarcula marismortui*.

However, little evidence has been obtained for massive binding of K^+ inside the cytoplasm of other halophilic Archaea. In lysed freeze-thawed cell pastes of *Halobacterium salinarum* potassium ions were found to diffuse freely in equilibrium dialysis experiments, while the intracellular Mg^{2+} was mostly bound. Moreover, direct measurement of K^+ concentrations in these pastes by a K^+ -specific electrode (measuring ion activity rather than concentration) yielded values comparable to the total K^+ concentration as measured by atomic absorption. It was thus concluded that K^+ largely exists in a free state inside the cells, and is prevented from leaving the cell by the intrinsic permeability properties of the cell envelope (Lanyi and Silverman, 1972).

Another approach that led to similar conclusions was the measurement of freezing transitions by differential scanning calorimetry, determining the apparent latent heat of fusion of thick pastes of *Halobacterium salinarum*, using pastes of cells lysed by freezing and thawing as a control. These experiments were designed to detect any physico-chemical abnormalities of the cell water. All differences observed between internal and external solutions and between whole and lysed bacteria could be attributed to the different effects of the two salts on the freezing behavior, and no evidence was found of any physico-chemical abnormality in the halobacterial cytoplasm. Similarly, ^{39}K nuclear magnetic resonance measurements of intact cells yielded did not support any massive binding of K^+ in *Halobacterium* (Brown and Sturtevant, 1980).

Additional evidence for the absence of "abnormal" behavior of the solution in the cytosol came from measurements of the heat of dilution of thick pastes of *Halobacterium salinarum* lysed by mixing with 40 times their volume of water in a microcalorimeter. Frozen-thawed pastes gave endothermic values some 18% greater than those obtained with intact bacteria. However, when a correction was applied for the heat of mixing of intracellular salts with extracellular NaCl such as occurs when the bacteria lyse, the difference between the whole and disrupted organisms was largely eliminated in exponential-phase cells (Brown and Duong, 1982).

4.4 Properties of proteins from halophilic Archaea

4.4.1 SALT REQUIREMENT AND TOLERANCE OF HALOPHILIC ARCHAEAL ENZYMES

The report by Robinson and Katznelson (1953) on the aspartate-glutamate transaminase from *Halobacterium salinarum* was probably the first paper in which the halophilic character of enzymes from halophilic Archaea was documented. This work was rapidly followed by studies on a variety of other enzymes from the same organism (Baxter and Gibbons, 1954, 1956). In all cases high salt concentrations were required for optimal activity and stability of the enzymes studied. In the absence of salt activity was irreversibly lost.

Since these early days a long list of enzymes from different species of halophilic Archaea have been studied [see e.g. the surveys given by Bayley and Morton (1978), Dundas (1977), Hochstein (1988), Kushner (1985, 1988), Lanyi (1974), and Larsen (1967)]. In many cases the enzymes were found to be more active in the presence of potassium chloride than of sodium chloride. An early example is the glycerol dehydrogenase of *Halobacterium salinarum* studied by Baxter and Gibbons (1954), which was inhibited above 1.5 M NaCl, but activities kept rising with increasing KCl concentrations up to values of 4 M. Such a behavior is understandable in view of the finding that K^+ is intracellularly the dominant cation (see section 3.1). Other enzymes were found to respond equally well to NaCl and KCl; however, effective allosteric regulation often depends on the presence of KCl rather than NaCl (Kushner, 1985). Amino acids incorporation by a cell-free system of *Halobacterium salinarum* ribosomes (measuring incorporation of ^{14}C -labeled phenylalanine in the presence of polyuridylic acid as template) had an absolute requirement for high potassium concentrations. Optimal activity was measured at 3.8 M KCl, 1 M NaCl, 0.4 M NH_4Cl , and 20–40 mM Mg^{2+} . To stabilize the ribosomes at least 3 M KCl and 0.1 Mg^{2+} were needed (Bayley and Griffiths, 1968). Thus, optimal conditions for this complex process, involving many separate enzymatic reactions, reflect the environment in which the process takes place in the intact cell. However, the cell surface glycoprotein, responsible for maintaining the shape of the cell and being exposed to the high NaCl concentrations outside the cell, specifically requires Na^+ for stabilization. When suspended in distilled water halophilic Archaea which possess a cell wall built of such a glycoprotein will lyse due to the denaturation and dissolution of the cell wall (Mohr and Larsen, 1963).

The salt requirements of halophilic enzymes for stability and for activity are not necessarily identical. In general, the best stabilizing salts are the “salting out” salts that decrease protein solubility and increase protein stability. “Salting-in” salts that directly interact with the polypeptide chain inactivate halophilic enzymes (Ebel *et al.*, 1999) (see also Figure 2). The behavior of different salts generally coincides with the lyotropic Hofmeister series (Lanyi, 1974).

The high solubility of halophilic proteins in the presence of high salt concentrations and the often irreversible denaturation at suboptimal salt concentrations has caused considerable problems in the purification of enzymes, which should preferentially be

performed in the constant presence of high salt concentrations. Methods that have successfully been applied include exclusion, hydrophobic, hydroxylapatite, and affinity chromatography or salting-out mediated chromatography (Eisenberg *et al.*, 1992). In selected cases it has also been possible to perform the purification of an inactive form of the enzyme at low salt using standard techniques, followed by a controlled renaturation and restoration of activity by increasing medium salinity.

It was already mentioned that the light-driven primary ion pumps bacteriorhodopsin and halorhodopsin are unusual among the proteins of the halophilic Archaea in their lack of any requirement for salt for stability and activity. Other protein structures found in certain species (*Halobacterium salinarum*, *Haloferax mediterranei* and a few others) that do not depend on salt for structural intactness are the gas vesicles that confer buoyancy to the cells.

4.4.2 MAINTENANCE OF PROTEIN STRUCTURE AND ENZYMATIC ACTIVITY AT HIGH SALT CONCENTRATIONS

The presence of high concentrations of solute ions is generally devastating to proteins and other macromolecules. It causes aggregation or structural collapse of proteins because of enhancement of hydrophobic interactions, it interferes with essential electrostatic interactions within or between macromolecules because of charge shielding, and it reduces the availability of free water below that required to sustain essential biological processes because of salt ion hydration (Dennis and Shimmin, 1997; Zaccai and Eisenberg, 1991). Therefore any discussion on the special properties of the proteins from halophilic Archaea that enable them to maintain solubility and enzymatic activity at the high *in situ* salt concentrations should start with an examination of the amino acid composition of these proteins. When analyzing the amino acid composition of the proteins of the *Halobacteriaceae*, either in bulk or in specific isolated proteins, the obvious features as compared to the proteins from non-halophilic microorganisms are: 1. a large excess of the acidic amino acids glutamate and aspartate, 2. a low content of the basic amino acids lysine and arginine, and 3. a low content of hydrophobic amino acid residues, which is often offset by an increased content of the borderline hydrophobic amino acids serine and threonine (Lanyi, 1974). The high content of acidic side groups was already recognized in 1970 during analyses of the bulk protein of *Halobacterium* and *Halococcus* (Reistad, 1970). The malate dehydrogenase of *Haloarcula marismortui* has 10.4 mol percent excess of acidic residues, and the cell envelope glycoprotein of *Halobacterium salinarum* even 19-20 mol percent.

It has been argued that the excess of acidic residues may be a major factor determining the halophilic character of the proteins: excess of negative charges on the protein surface makes the structure unstable because of the mutual repulsion of these side groups. Only when high concentrations of cations are added to shield the negative charges can the protein maintain its proper conformation required for structural stability and enzymatic activity. Shielding of negative charges by cations undoubtedly plays an important part in the effects of salt on the enzymes and other proteins of the halophiles. A theoretical analysis of the contribution of electrostatic interactions in *Haloarcula marismortui* ferredoxin and malate dehydrogenase shows that the repulsive interactions be-

tween the acidic residues at the protein surface are a major factor in the destabilization of halophilic proteins in low-salt conditions (Elcock and McCammon, 1998). However, Lanyi (1974) and Lanyi and Stevenson (1970) argued that all the effects of salts cannot be due to charge-shielding action. Maximal electrostatic charge shielding would be reached already in about 0.1 M salt or 0.5 M at most, and in even much lower concentrations of divalent cations. Another good reason for increasing the content of acidic amino acids may be the fact that glutamate has the greatest water binding ability of any amino acid residue. This may have important implications when considering the need of any functional protein to maintain a proper hydration shell.

The requirement for extremely high salt concentrations for structural stability of the proteins can probably be attributed to the low content of hydrophobic residues and the accordingly weak hydrophobic interactions within the protein molecules. High salt is then needed to maintain the weak hydrophobic interactions. Entropy increases when non-polar groups turn away from the water phase and interact with each other to form hydrophobic bonds. These interactions seem to be driven more by an avoidance of water than by an active attraction between the non-polar molecules (Lanyi, 1974). At higher concentrations of salt new hydrophobic interactions are formed which have insufficient stability in water, and the molecule assumes a more tightly folded conformation.

The possible involvement of the weak hydrophobic bonds in the salt requirement of the halophilic proteins appears from the finding that certain enzymes from halophilic Archaea show cold lability: their maximal stability is reached at temperatures greater than 0 °C and decreases at lower temperatures. The effect may be considered in terms of water structure: at lower temperature the size of the cluster of water molecules is increased, and hydrophobic groups can interact more easily, breaking the hydrophobic bonds (Lanyi, 1974).

Detailed studies of the malate dehydrogenase from *Haloarcula marismortui* have contributed much valuable information on the possible mechanisms involved in their halophilic behavior. The enzyme has been intensively investigated using techniques such as velocity sedimentation, light scattering, neutron scattering, and circular dichroism measurements, to obtain information on the structural changes occurring as a function of changing salt concentrations and the hydration properties of the protein (Eisenberg, 1995; Eisenberg and Wachtel, 1987; Mevarech and Neumann, 1977; Pundak and Eisenberg, 1981; Pundak *et al.*, 1981). These studies showed that the halophilic properties of the enzyme are related to its capacity of associating with unusually high amounts of salts. Different modifications have been artificially introduced in the enzyme by site-directed mutagenesis. Studies of the functioning and the stability of the native enzyme and these mutants led to a thermodynamic "solvation-stabilization model" in which the halophilic protein has adapted to bind hydrated ions cooperatively via a network of acidic groups on its surface (Ebel *et al.*, 1999) (Figure 2).

X-ray diffraction studies on crystals of halophilic malate dehydrogenase and ferredoxin of *Haloarcula marismortui* have added much important information (Dym *et al.*, 1993; Frolov *et al.*, 1996). These studies enabled for the first time to obtain a picture how the carboxylic groups on the acidic residues are used to sequester, organize, and arrange a

tight network of water and hydrated K^+ ions at the surface of the protein and to form an unusually large number of internal salt bridges with strategically located basic amino acids residues to provide internal structural rigidity of the protein. These salt bridges appear to be important determinants in the stabilization of the three-dimensional structure of halophilic proteins. As expected, most acidic residues map to the protein surface, and are believed to coordinate a network of water and hydrated salt ions at the protein solvent interface. The negative charges of the carboxylic acid groups on the surface of the protein are shielded from each other by intervening solvent molecules.

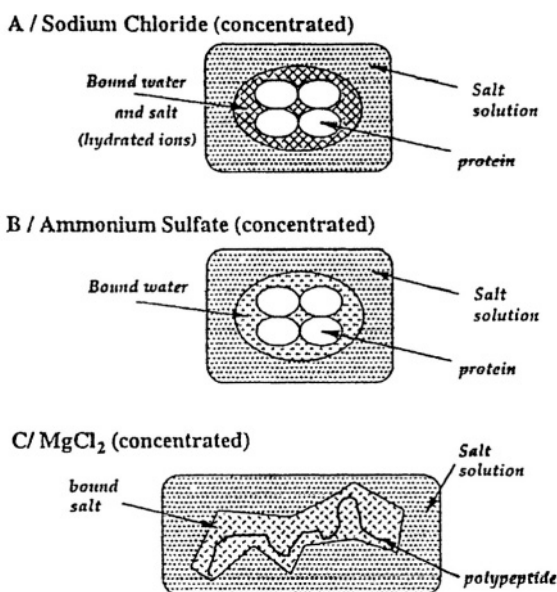


Figure 2. Salt ion effects (at molar concentrations) on *Haloarcula marismortui* malate dehydrogenase stability, solubility, and solvation. (A) At high concentrations of NaCl (above ca. 2 M) the stabilization occurs via hydrated ion binding to the folded tetramer. The same situation holds for high concentrations of KCl, or in $MgCl_2$ concentrations of 0.5-1.3 M. (B) At high concentrations of $(NH_4)_2SO_4$ (above ca. 1 M) the classical salting-out model is valid. (C) At high concentrations of $MgCl_2$ the protein unfolds, and the classical salting-in model may be valid (from Ebel *et al.*, 1999, reproduced with permission).

Comparison of the *Haloarcula marismortui* ferredoxin with the plant-type 2Fe-2S ferredoxin showed that the surface of the halophilic protein is coated with acidic residues except for the vicinity of the iron-sulfur cluster, and that it contains two additional helices near the N-terminus that form a separate hyperacidic domain, postulated to provide extra surface carboxylates for solvation. Bound water molecules on the protein surface have on the average 40% more hydrogen bonds than in a typical non-halophilic protein crystal structure. These water molecules are thus tightly bound within the hy-

dration shell by protein-water and water-water hydrogen bonds and by hydration of interspersed K^+ ions (Frolow *et al.*, 1996). Not all proteins from halophilic Archaea show the above-discussed properties. Dihydrofolate reductase of *Haloferax volcanii*, the third enzyme characterized by X-ray crystallography, does not exhibit strikingly halophilic features (Pieper *et al.*, 1998).

The successful crystallization of ribosomal particles from *Haloarcula marismortui*, enabling to obtain structural information at 2.9 Å resolution (Francheschi *et al.*, 1994) may also be expected to yield a wealth of new information on the structure-function relationships of halophilic proteins. This may include information toward an understanding of the protein-nucleic acid interactions, which are essential for the performance of biological functions. The existence of meaningful protein-nucleic acid interactions in physiological concentrations of 4 to 5 M KCl constitutes as yet an unsolved enigma, worth an intensive investigation (Ebel *et al.*, 1999).

4.5 Epilogue

In the previous sections our present understanding of the specific adaptations of halophilic Archaea to life at high salt concentrations was summarized. These microorganisms and their enzymatic systems have evolved particular properties, enabling them to function in the presence of the high salt concentrations found not only in the medium, but also inside the cell. The evolutionary implications of the development of these halophilic proteins have been discussed in depth by Dennis and Shimmin (1997).

The adaptation of the cellular machinery to function in the presence of saturated KCl concentrations implies a limited flexibility of the cell with respect to its ability to adapt to a wide range of - often rapidly changing - salt concentrations. Microorganisms using organic compatible solutes show a much higher adaptability to changing salinities. In this respect it is of some interest to note that it was recently shown that certain alkaliphilic halophilic Archaea, in addition of possessing high intracellular salt concentrations, may contain an organic osmotic solute as well: 2-sulfotrehalose (Figure 3). When grown in defined media lacking yeast extract this novel compound was accumulated by *Natronococcus occultus* at concentrations of up to 0.9 M. However, in rich media containing yeast extract, the use of inorganic ions is preferred over *de novo* synthesis of sulfotrehalose (Desmarais *et al.*, 1997). Whether the use of organic compatible solutes confers a higher degree of flexibility to these cells with respect to life in changing salt concentrations remains to be determined.

The finding of organic osmotic solutes in some alkaliphilic halophilic Archaea under certain growth conditions, as stated above, does not change our general view of haloadaptation of the members of the *Halobacteriaceae*, based on maintenance of extremely high intracellular ionic concentrations and the adaptation of the intracellular machinery to function optimally in the presence of these high salt concentrations. The molecular mechanisms involved in this adaptation have become increasingly clear during recent years. With the improvements in the techniques used, notably X-ray diffraction of crystals of isolated enzymes and site-directed mutagenesis studies enabling the elu-

cidation of the importance of specific amino acid residues in the maintenance of enzyme structure and activity, it may be expected that much additional information will become available in the near future, leading to a more in-depth understanding of the functioning of these fascinating microorganisms.

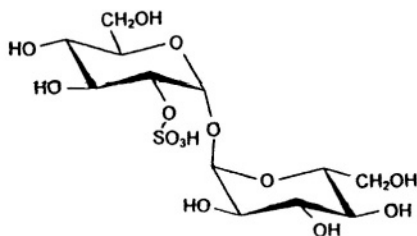


Figure 3. The structure of 2-sulfotrehalose.

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CHAPTER 5

ADAPTATION OF THE HALOTOLERANT ALGA *DUNALIELLA* TO HIGH SALINITY

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Abstract

The unicellular halotolerant green alga *Dunaliella* is unique in its ability to adapt to most hypersaline ecosystems. *Dunaliella* maintains osmotic balance at high salinities by synthesis of intracellular glycerol. Regulation of glycerol synthesis involves activation of plasma membrane protein kinases. *Dunaliella* effectively excludes Na^+ ions via two putative Na^+ transporters. High salinity induces enhanced synthesis of two major plasma membrane proteins: a transferrin-like protein and a carbonic anhydrase, which mediate acquisition of Fe^{3+} ions and CO_2 , whose availability is greatly diminished at high salinity. Both proteins are salt-resistant and are structurally modified with respect to mesophilic protein counterparts.

5.1 Introduction

Many species of algae have adapted to different levels of salinity, but only a few flourish in the most extreme hypersaline environments such as the Dead Sea in Israel. The green halotolerant alga *Dunaliella* is among the very few which thrives in hypersaline niches occupied almost exclusively by halophilic archaeobacteria (Chapter 4) and it is the dominant photosynthetic eukaryote in most natural and artificial hypersaline lakes around the world. In contrast to obligate halophilic archaeobacteria, which survive only in high salt, most *Dunaliella* species can adapt to an exceptionally wide range of salt concentrations, from less than 100 mM to saturated (5.5 M) NaCl solutions (Avron, 1992).

What are the features that enable microorganisms to survive in hypersaline environments? Halophilic archaeobacteria and halotolerant eukaryotes such as *Dunaliella* utilize different strategies: While halophilic archaeobacteria accumulate from the external medium potassium salts as an internal osmoticum, halotolerant eukaryotes synthesize a variety of organic solutes, which are compatible with protein function at

high concentrations to balance their internal osmolarity. Osmocompatible solutes (Chapter 9) include derivatives of amino acids (glycine betaine, proline), polyols (glycerol, mannitol) and carbohydrates (sucrose). The accumulation of high salt in archaeobacteria created an evolutionary pressure to evolve salt-resistant proteins and indeed, most cytoplasmic proteins in halophilic archaeobacteria are salt-dependent proteins (Chapter 4). In contrast, cytoplasmic proteins from halotolerant glycophytes resemble mesophilic proteins, which are salt-sensitive. A special case are extracellular proteins of halotolerant glycophytes which are exposed to high salt concentrations and therefore are expected to resemble halophilic archaeobacterial proteins (Figure 1). In this regard, *Dunaliella* is an intriguing example, since its extracellular proteins should remain functional both at high salt and at low salt, to meet the natural living conditions of this species. Indeed, as will be described below, two extracellular proteins of this alga share an unusual functional plasticity to function in widely varying salt concentrations.

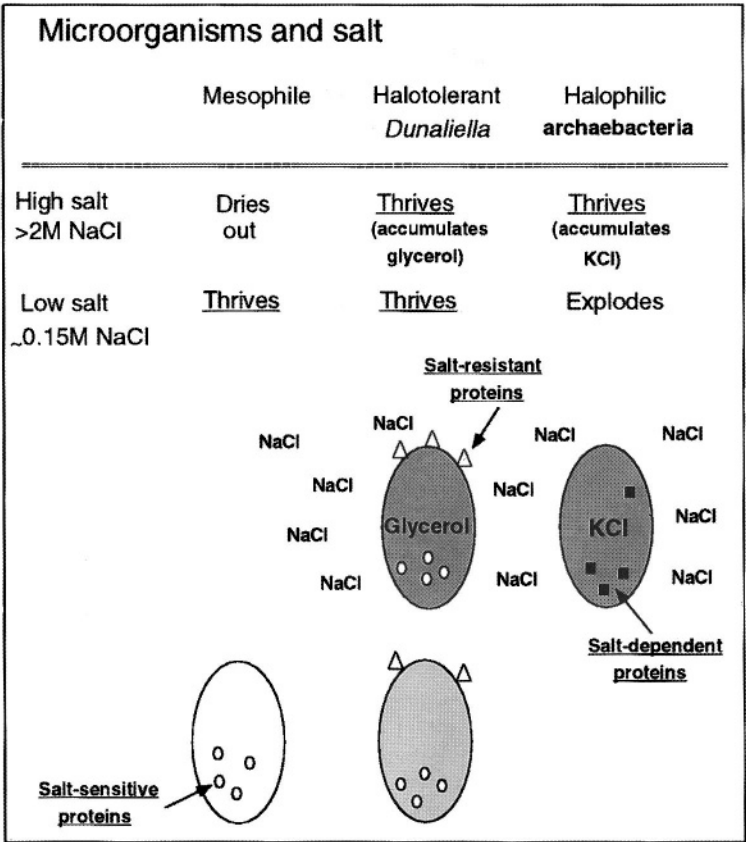


Figure 1. Different strategies of adaptation to hypersaline environment of halophilic archaeobacteria and halotolerant *Dunaliella*.

This review summarizes the basic features, which enable *Dunaliella* to adapt to hypersaline environments. Previous extensive reviews concerning the physiological and biochemical adaptation of *Dunaliella* to high salinity and its economic potential have already been published (Ginzburg, 1987; Borowitzka and Borowitzka, 1988; Avron and Ben Amotz, 1992; Pick, 1998).

5.2 Osmoregulation - why glycerol?

At high salinity *Dunaliella* accumulates massive amounts of glycerol and the level of intracellular glycerol was found to be proportional and osmotically equivalent to the external NaCl concentration, reaching about 8 M or 55% of the cell weight at saturated NaCl (Ben Amotz and Avron, 1973). No significant changes in intracellular K^+ , Na^+ and Cl^- contents were detected at varying salt concentrations indicating that glycerol is the only osmotic element in *Dunaliella* (Pick *et al.*, 1986; Avron, 1986). Only at elevated temperatures *Dunaliella* accumulates also significant amounts of sucrose (Mueller and Wegmann, 1978).

The response of *Dunaliella* to osmotic variations has been extensively reviewed (Avron, 1992) and will only be briefly described here. It involves gross structural and metabolic changes as depicted schematically in Figure 2: When subjected to a hyperosmotic shock (salt stress) the wall-less *Dunaliella* cells rapidly shrink followed by synthesis of glycerol which increases the internal osmolarity until the cells resume their original volume. The exact opposite response occurs following a hypoosmotic (dilution) shock, namely, rapid swelling followed by a decrease in internal glycerol and volume resumption.

Glycerol biosynthesis begins almost immediately following salt stress and the process does not depend on *de-novo* synthesis of new enzymes (Sadka *et al.*, 1989). The carbon sources for glycerol synthesis are photosynthetic CO_2 fixation and starch reserves within the chloroplast. Biosynthesis of glycerol involves two specific enzymes, which convert dihydroxyacetone-phosphate (DHAP) to glycerol: glycerolphosphate (GP) dehydro-genase and a unique GP phosphatase. In the opposite direction, glycerol is converted to DHAP by an NADPH-specific DHA-reductase and a DHA-kinase. It is not exactly clear how glycerol mobilization from starch and *vice versa* is being regulated and what prevents a futile recycling of glycerol. Each of the final enzymatic reactions includes one irreversible step (GP phosphatase and DHA kinase) and it was suggested that these enzymes are differentially regulated. Changes in metabolite levels following hyperosmotic shocks (increase in the ratio of F-1,6-P/F-6-P) indicated that phosphofructokinase may be a checkpoint enzyme in the activation of glycerol synthesis. The synthesis and reassimilation of glycerol take place mostly within the chloroplast and only the terminal enzymatic steps that are catalyzed by DHA-reductase and possibly GP-phosphatase are localized in the cytoplasm.

The utilization of glycerol as a major osmotic element is not unique to *Dunaliella* and is common in yeast and fungi and in a few other algal species but not in prokaryotes. Glycerol is characteristic but not exclusive to eukaryotic microorganisms which thrive in extreme hypersaline environments (Borowitzka, 1985).

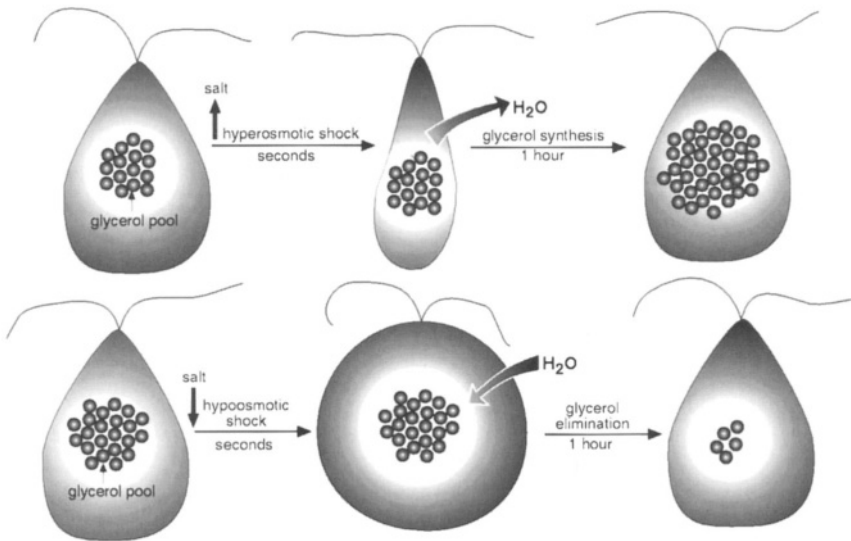


Figure 2. Volume changes and glycerol mobilization in response to osmotic changes in *Dunaliella*.

What makes glycerol such an effective osmotic element at high salinities? Several reasons may be considered: First, the high solubility of glycerol cannot be matched by most other osmocompatible solutes. Second, glycerol is chemically inert and therefore non-toxic. Third, glycerol is an end-product metabolite, and therefore its accumulation is unlikely to offset major metabolic pathways. Fourth, the energetic cost of glycerol synthesis from starch is relatively low (Chitlaru and Pick, 1991) and it does not depend on the availability of nitrogen. However, glycerol suffers from one major limitation as a perfect osmotic element which is its high permeation across biological membranes. What enables *Dunaliella* to maintain glycerol within the cells? It was demonstrated that *Dunaliella* plasma membranes are exceptionally impermeable to glycerol (Enhuber and Gimmler, 1980; Brown *et al.*, 1982), suggesting that they may have a unique structure. Indeed, *Dunaliella* plasma membrane composition is quite unusual: the overall polar lipid content is relatively low, comprising only approximately 30% of the total carbon content (Pick, unpublished observations). The lipid composition is also

unusual consisting of a major unorthodox diethylglyceroether-trimethyl-homoserine and of a relatively high content of sterols and sterol epoxides relative to phospholipids (Sheffer *et al.*, 1986; Peeler *et al.*, 1989). Even though glycerol is an excellent osmocompatible solute, it may limit enzymatic activities at very high concentrations. In fact, some enzymes from *Dunaliella* seem to be specifically adapted to function in high glycerol: The chloroplast ATP synthase ($\text{CF}_0\text{-CF}_1$) of *D. bardawil*, is stimulated by 20% glycerol, in contrast to $\text{CF}_0\text{-CF}_1$ of higher plants which is strongly inhibited by glycerol (Finel *et al.*, 1984). The structural modifications that enable *Dunaliella* $\text{CF}_0\text{-CF}_1$ to function in high glycerol are not clear.

Glycerol may have an additional ecologically-important function, namely, to enable species of *Dunaliella* to survive at subzero temperatures in hypersaline lakes, probably by acting as effective antifreeze (Watanuki *et al.*, 1987; Franzmann, 1991).

5.3 Volume changes and osmosensing

A fascinating quality of *Dunaliella* is its ability to withstand remarkable osmotic changes. Since all *Dunaliella* species are devoid of a rigid cell wall they respond to osmotic changes by changes in cell volume. *Dunaliella* can be exposed either to hyperosmotic or to hypoosmotic shocks of up to 3-5 fold with respect to the initial osmolarity, resulting in approximately two-fold changes in cell volume within seconds (Ehrenfeld and Cousin, 1984; Weiss and Pick, 1990). What confers such a high plasticity to the plasma membrane? Electron-microscopic analyses revealed ultrastructural changes taking place within the plasma membranes shortly after osmotic shocks indicative of rapid infolding following hyperosmotic stress and of fusion with a sub-population of small cytoplasmic vesicles following dilution shocks (Maeda and Thompson, 1986; Einspahr *et al.*, 1988). These results have indicated that *Dunaliella* possesses a membrane reservoir which enables a rapid increase or decrease, respectively, in cell volume which prevents lysis of the cells. Such a mechanism can confer a selective advantage to an organism which is exposed to periodic changes in osmolarity such as *Dunaliella*.

The osmotic volume changes induce structural and enzymatic activations within the plasma membrane which have been implicated with triggering glycerol mobilization (reviewed by Avron, 1992). They include : (i) transient lipid reorganizations measured with spin-probe lipid analogs (Fontana and Haug, 1982; Curtain *et al.*, 1983); (ii) differential hydrolysis of plasma membrane phospholipids phosphatidylcholine and phosphatidyl-inositol-diphosphate, indicative of activation of distinct types of phospholipase C following hyperosmotic or hypoosmotic shocks, respectively; (iii) transient activation of a 74 kDa plasma membrane protein kinase which resembled mitogen-activated-protein (MAP) kinase kinases in animal cells in its substrate specificity (Chitlaru *et al.*, 1997). The MAP kinase cascade is known to be involved in osmotic signaling in yeast and mammalian cells (Maeda *et al.*, 1994; Han *et al.*, 1994). It has also been demonstrated that depletion of plasma membrane sterols blocked

glycerol production, whereas re-supplementation of exogenous sterols restored glycerol synthesis and volume recovery (Zelazny *et al.*, 1995).

These observations indicated that the osmosensing element in *Dunaliella* may be a protein kinase which is activated by lipid reorganizations involving specific sterols within the plasma membrane (Pick, 1998).

5.4 Ionic homeostasis and Na^+ elimination

Na^+ ions in *Dunaliella* are strictly regulated by active transport mechanisms which keep their internal concentration far from electrochemical equilibrium with the membrane potential (reviewed by Pick, 1992). Intracellular Na^+ ion concentrations are maintained around 10-30 mM over a wide range of external NaCl concentrations of up to 4 M, indicating the existence of an active Na^+ export mechanism in *Dunaliella* plasma membranes. However, the mechanism of Na^+ elimination in *Dunaliella* is not well understood. Katz *et al.* (1986) identified an amiloride-sensitive Na^+/H^+ antiporter activity in plasma membrane preparations of *D. salina*, which was originally implicated to be involved in Na^+ elimination. However, subsequent studies have revealed that the antiporter catalyzes Na^+ influx rather than Na^+ extrusion, and that it is activated by internal acidification or by hyperosmotic shocks (Katz *et al.*, 1991; Weiss and Pick, 1990). What is the mechanism of Na^+ extrusion, which enables *Dunaliella* to maintain such a low intracellular Na^+ concentration?

Kinetic studies in intact cells showed that Na^+ elimination in *Dunaliella* was insensitive to amiloride but was blocked by vanadate or diethylstilbestrol, typical inhibitors of "P-type" ATPases. These results suggested that a putative Na^+ -ATPase might carry out Na^+ extrusion in *D. salina* (Figure 8 in Pick, 1992). It may be noted that plasma membrane Na^+ -ATPases have already been identified in two marine algae (Balnokin and Popova, 1994; Shono *et al.*, 1996; Popova *et al.*, 1998). Very recently we obtained preliminary evidence for operation of a plasma membrane Na^+ -coupled electron transport system (A. Katz, unpublished observations). Based on these results we suggest that two distinct Na^+ extrusion systems operate in parallel in *Dunaliella* plasma membranes: a Na^+ -ATPase and an NADH-driven electron transport Na^+ pump (Figure 3). Thus, the Na^+ homeostasis mechanisms in *Dunaliella* are more similar to those in animal cells (primary plasma membrane Na^+ pumps and a Na^+/H^+ antiporter associated with pH regulation) and to certain bacteria (electron transport-driven Na^+ extrusion) than to plant cells which utilize H^+ -ATPases as their primary plasma membrane cation pumps.

5.5 pH homeostasis

The primary system for proton extrusion in plants and plant-like organisms is the plasma membrane vanadate-sensitive H^+ -ATPase. *Dunaliella salina* differs from higher plants, in that it utilizes primarily the Na^+/H^+ antiporter rather than a plasma membrane H^+ -ATPase for H^+ extrusion. The involvement of the Na^+/H^+ antiporter in pH regulation was demonstrated by massive influx of Na^+ ions in exchange for protons

elicited by internal acidification in *D. salina* cells (Katz *et al.*, 1991). This activity corresponded to the amiloride-sensitive Na^+/H^+ exchange activity previously identified in plasma membrane vesicles (Katz *et al.*, 1986). Conversely, the activity and protein level of the H^+ -ATPase in *D. salina* plasma membranes appear to be very low as revealed from the lack of ATP-dependent H^+ uptake activity in inside-out plasma membrane preparations and from immuno-analyses (Weiss *et al.*, 1989; Weiss and Pick, 1996). Thus, *D. salina* has adapted to utilize the large existing Na^+ gradient across the plasma membrane rather than ATP as the driving force to counterbalance internal acidification.

A different mechanism was activated in *D. salina* upon an alkaline stress induced by amines at high external pH. We have shown that cytoplasmic alkalization was re-

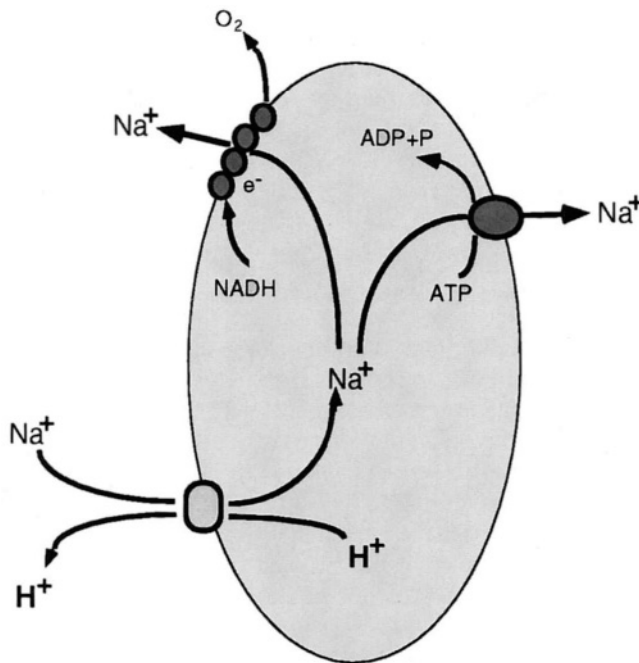


Figure 3: Putative Na^+ transporters involved in the homeostasis of cytoplasmic Na^+ concentration in *Dunaliella*.

lieved by compartmentation of amines from the cytoplasm into acidic vacuoles which contained large amounts of polyphosphates. The accumulation of amines within the vacuoles was associated with massive hydrolysis of polyphosphates and a consequent generation of protons, which neutralized the rise in pH. In this way cells could

accumulate and accommodate up to 500 mM (!) ammonia (Pick *et al.*, 1991; Pick and Weiss, 1991).

An exceptional example of pH homeostasis is the extremely acidophilic *D. acidophila* which grows optimally at pH 0-1 while keeping a neutral cytoplasmic pH (reviewed by Gimmmler and Weis, 1992). How does an organism maintain a pH gradient of 7 pH units (ten million folds) across its membrane? This is achieved by a combination of factors which decrease the permeability of the membrane to protons and an effective proton export mechanism. In contrast to *D. salina*, *D. acidophila* overexpresses a potent plasma membrane **H⁺-ATPase** that has been biochemically characterized and cloned. Sequence analysis of the gene revealed that the enzyme was a structurally modified homologue of higher-plant **H⁺-ATPase** possessing a positively charged cytoplasmic loop. This modification may be associated with adaptation to function in acidic media (Sekler *et al.*, 1991, 1994; Weiss and Pick, 1997). Similar to extreme acidophilic bacteria, *D. acidophila* maintained a positive-inside membrane potential and possessed a positive surface charge, factors which decrease the effective influx of protons into the cells. Another unusual feature of this extreme acidophile is the operation of a **K⁺/H⁺** symport mechanism at the plasma membrane (Gläser *et al.*, 1990). Thus, pH homeostasis in *D. acidophila* resembles neither other *Dunaliella* species nor higher plants (reviewed by Pick, 1992).

5.6 High salt-induced proteins in *Dunaliella*

Acclimation to high salinity in most halotolerant micro and macroorganisms is associated with activation of specific genes and synthesis of special enzymes. In *Dunaliella* the basic osmotic responses, namely, synthesis of glycerol and elimination of Na⁺ ions, do not depend on *de novo* synthesis of new enzymes, suggesting that both capacities are constitutive. However, adaptation to very high salt (3.5 M NaCl), leads to up-regulation of several genes and to the accumulation of specific proteins, mostly but not exclusively, within the plasma membrane. Cloning of the corresponding genes has led to clarification of the roles of the encoded proteins and to identification of rate-limiting steps for survival in hypersaline solutions (Table 1).

TABLE 1: Salt-induced proteins/genes in *Dunaliella*.

gene/ Protein	MW kDa	Sub-cellular localization	Gene cloned	Function	Ref*
ttf	150	PM	+	Fe uptake	1
dc	60	PM	+	Carbonic anhydrase	2
fae1	70	ER	+	Fatty acid elongase	3
sig37	37	Chloroplast	+	?	4
p30	30	PM	-	Na ⁺ /H ⁺ antiporter	5

PM-plasma membrane, ER- endoplasmic reticulum; *1- Fisher *et al.*, 1997,1998; 2- Fisher *et al.*, 1996; 3. Fisher, 1998; Azachi and Zamir; unpublished observations, 4. A. Katz, unpublished observations, 5. Katz *et al.*, 1994.

5.6.1 A TRANSFERRIN-LIKE PROTEIN MEDIATING IRON UPTAKE

High salt induced the accumulation of two major plasma membrane proteins in *D. salina* of 150 kDa and 60 kDa (Sadka *et al.*, 1991; Fisher *et al.*, 1994). Cloning and sequencing of the ttf gene revealed that it consisted of three internal repeats, each manifesting homology to mammalian transferrins. The protein was termed TTF for triplicated transferrin. Following the established role of transferrins in Fe metabolism in animals, a search to clarify the role of TTF revealed that TTF was induced also under conditions of Fe limitation and suppressed by Fe supplementation and that the rise in level of TTF in plasma membranes was correlated with enhanced Fe-uptake activity in intact cells. These results suggested that TTF was associated with iron acquisition (Fisher *et al.*, 1997, 1998). The similar effect of Fe limitation and high salinity on induction of TTF indicated that high salinity limited the availability of iron ions to *Dunaliella* cells. Thus, Fe availability appears to be a rate-limiting step for cell proliferation at hypersaline environments (Fisher *et al.*, 1998).

More direct confirmation for the role of TTF was obtained from analysis of Fe^{3+} binding to solubilized TTF (M. Ortal-Shwartz, unpublished observations) which closely resembled the characteristics of Fe uptake in intact cells (Fisher *et al.*, 1998) both were dependent on the presence of bicarbonate anions and exhibited high affinity ($K_a = 10^{-18} - 10^{-19}$ M) and high specificity for Fe^{3+} ions. These characteristics were remarkably similar to Fe^{3+} binding to mammalian transferrins. Thus, TTF is the first reported plant transferrin-like protein which functions in Fe acquisition.

A unique feature of TTF was its resistance to high salinity: Fe^{3+} binding and uptake were resistant even to 3.5 M NaCl (Figure 4A). The special significance of this feature will be discussed in the next section.

5.6.2 A PERIPLASMIC CARBONIC ANHYDRASE ENHANCING CO_2 ACQUISITION

The 60 kDa protein which was induced by high salt in *Dunaliella* plasma membranes (Fisher *et al.*, 1994) was purified and cloned. Sequence analysis revealed that it was internally-duplicated and homologous to carbonic anhydrase (CA), (Fisher *et al.*, 1996). The purified protein had CA activity confirming that it was an extrinsic *Dunaliella* carbonic anhydrase (Dca). Dca was induced also at alkaline pH and depletion of CO_2 , in parallel with induction CA activity. Thus, high salinity induced in *Dunaliella* symptoms of CO_2 deprivation consistent with earlier studies (Latorella and Vadas, 1973; Booth and Beardall, 1991). What is the relationship between high salinity and CO_2 availability? High salinity decreases the solubility and diffusion of CO_2 (Booth and Beardall, 1991) and also decreases the dissociation constants for carbonate and bicarbonate (Saas and Be-Yaakov, 1977). These factors are expected to decrease the steady-state concentrations of medium CO_2 in hypersaline media leading to its rapid depletion under conditions of extensive photosynthetic CO_2 consumption.

Similar to TTF, also *Dunaliella* CA was found to be highly resistant to high salt: the enzyme maintained high activity in low as well as in high salt, in contrast to the *Chlamydomonas reinhardtii* CA which was strongly inhibited by high salt (Figure 4B).

The observation that two extracellular proteins in *Dunaliella*, TTf and Dca, which fulfill different roles, are not inhibited but rather stimulated by high salt, is unique. They differ both from mesophilic enzymes which are inhibited by high salt, and from halophilic enzymes which are dependant on high salt (see Figure 1). This raises the question whether these proteins possess common structural features associated with their functional plasticity. Indeed, *Dunaliella* TTf and Dca share two common structural features: First, both consist of internal repeats (Figure 5): TTf consists of three rather than two internal repeats, typical to mammalian transferrins, whereas Dca contains two internal repeats in comparison to the single copy in mammalian CA and to the heterotetramer (**2 α** , **2 β**) of *C. reinhardtii* periplasmic CA (Fujiwara *et al.*, 1990). Second, both enzymes contain a large excess of acidic over basic amino acids and have lower isoelectric points in comparison to mesophilic counterparts (Pick, 1998).

Notably, the only other reported case of a 150 kDa transferrin-like protein is a marine organism, the crab *Cancer magister* (Heuberts *et al.*, 1982). Thus internal repeats may be associated with protein adaptation to hypersaline environments. The excess of negative charge is a hallmark of proteins from halobacteria and is believed to stabilize proteins at high salt concentrations by increasing their hydration level (Dym *et al.*, 1995; Frolow *et al.*, 1996). Conclusive establishment of the functional significance of internal repeats and of excess negative charge in TTf and in Dca will require detailed structural analyses and directed structural modifications of the corresponding genes.

5.6.3 Na^+/H^+ ANTIPORTER

Indications that the level of the Na^+/H^+ antiporter in *D. salina* plasma membranes (Katz *et al.*, 1986) may be salt-regulated were reported. Adaptation of *D. salina* cells to high NaCl was associated with a large increase in Na^+/H^+ antiport activity in plasma membrane preparations (Katz *et al.*, 1992). High salt also increased the level of two plasma membrane polypeptides, of 30 kDa and 50 kDa, which were labeled by a photoaffinity analog of amiloride, 2'-methoxy-5'-nitrobenzamil, that specifically inhibited the Na^+/H^+ antiporter (Katz *et al.*, 1994). The salt-induced accumulation of the antiporter implied that it is involved in Na^+ homeostasis.

5.6.4 FATTY ACID ELONGASE

A 70 kDa putative fatty-acid-elongase-encoding gene (fael) has been cloned from a salt-induced cDNA library of *D. salina*. Sequence analysis of fael revealed a striking homology to higher plant fatty acid elongases (Fisher *et al.*, 1998). High salt was found to enhance the expression of the corresponding 70 kDa protein and fatty-acid-elongase activity in the endoplasmic reticulum membrane fraction (M. Azachi and A. Zamir, unpublished observations). The functional role of this fatty-acid-elongase is still not clear, however, analysis of the fatty acid composition from plasma membrane polar lipids revealed a distinct increase in the length of fatty acids from high salt adapted cells (M. Azachi and A. Zamir, unpublished observations). These results indicated that

the salt-induced fatty-acid elongase in *Dunaliella* might be associated with modification of the plasma membrane lipid composition.

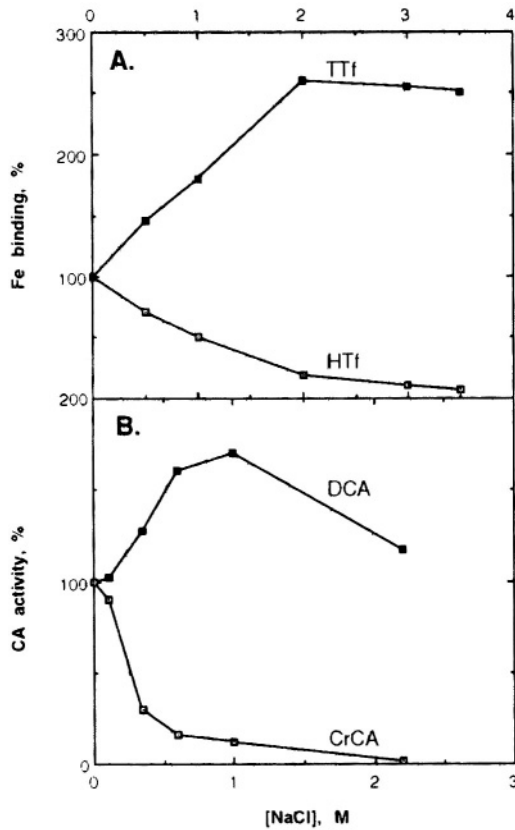


Figure 4: Effect of salt on the activity of *Dunaliella* extracellular enzymes in comparison to mesophilic enzyme counterparts. A. Fe binding to transferrins, B. Carbonic anhydrase (CA) activity. TTF- *Dunaliella* triplicated transferrin, HTF- human transferrin, DCA, *Dunaliella* carbonic anhydrase, CrCA- *C. reinhardtii* carbonic anhydrase.

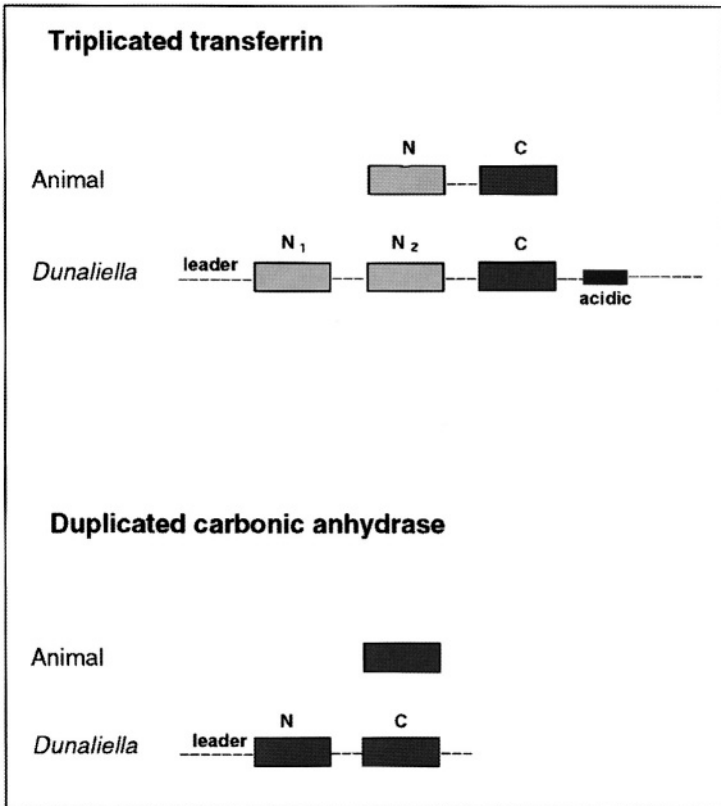


Figure 5: Internal repeats in *Dunaliella* extracellular proteins in comparison to animal homologues.

5.6.5 GENES OF UNKNOWN FUNCTION.

Another gene cloned from a salt-induced library of *D. salina*, encoding a salt-induced 37 kDa protein (sig37), was identified. A corresponding protein was immunolocalized in chloroplast membrane fractions. Sequence analysis of the gene revealed a partial homology to glucosyl-transferases from *Rhizobium*, however no functional enzymatic activities were identified to confirm this prediction (A. Katz, unpublished observations).

5.7 Summary and future perspectives

Studies aimed to clarify how *Dunaliella* adapts to high salinity yielded several important conclusions: (i) it led to identification of rate-limiting steps for survival in hypersaline environments (e.g. reduced availability of CO₂ and iron ions); (ii) it clarified cellular mechanisms associated with survival at high salinity (accumulation of glycerol, Na⁺ elimination, membrane plasticity, induction of proteins associated with CO₂ and iron acquisition); (iii) it focused attention on a novel class of proteins which can function either in the presence or absence of high salt. Structure-function analyses of these proteins may reveal new structural elements responsible for the functional plasticity in varying salinities; and finally (iv) *Dunaliella* may serve as a source of genes for transformation of plants in attempt to generate transgenic crop plants with improved salt-tolerance.

A question that should be addressed is to what extent can *Dunaliella* be considered a model organism of salt tolerance for higher plants. Previous biochemical and molecular studies demonstrated that *Dunaliella* resembled higher plants in its basic metabolism, photosynthetic activities and in many responses to stress conditions. For example, *Dunaliella* responded to photoinhibition by enhanced turnover of the PSII D₁ protein (Gounaris et al., 1987), by activation of the xanthophyll deepoxidation cycle (Levi et al., 1993) and by induction of a carotene-biosynthesis-related (Cbr) (Lers et al., 1991), a homologue of early-light-induced (ELIP) proteins in higher plants. Several proteins from *Dunaliella* are modified versions of higher plant homologues which are specifically adapted to function under extreme environments: for example, Dca, the salt-resistant carbonic anhydrase is homologous to *C. reinhardtii* periplasmic CA (Fisher et al., 1996), the glycerol-activated CF₀-CF₁ resembles in its function and subunit composition the higher plants chloroplast ATP synthase (Finel et al., 1984), and the plasma membrane H⁺-ATPase of *D. acidophila* and *D. salina* are modified versions of the higher plant enzymes and have distinct structural features (Weiss et al., 1997). These are examples of evolutionary adaptations of proteins to function under extreme conditions which justify consideration of *Dunaliella* as a model for salt tolerance. However, *Dunaliella* differs from higher plants in several biochemical and structural features: the massive synthesis of glycerol, the exceptional capacity for elimination of Na⁺ via two putative primary Na⁺ transporters and the presence of a transferrin-like protein, may be unique to *Dunaliella* and possibly to other algal species. Future studies will tell whether these and additional proteins associated with salt tolerance are indeed unique to *Dunaliella* or whether they are modifications of mesophilic enzymes shaped by evolution to improve survival in hypersaline environments.

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CHAPTER 6

MANGROVES

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Abstract

Mangroves, typical ecosystems of coastlines in the tropics and subtropics, are among the most productive ecosystems on earth. Primary productivity is based on mangrove trees (60 – 80 %), macroalgae (15 – 20 %) and microbial mats (5 - 20 %). Stress conditions of mangroves are given by highly variable substrate salinity, desiccation, irradiance, temperature. The trees in this ecosystem are adapted by special morphological characteristics, salt partitioning, and features of photosynthesis, water-use-efficiency and photoprotection. Macroalgae are extremely shade adapted and some of them are desiccation tolerant.

6.1 Mangrove Ecosystems

6.1.1 PHYTOGEOGRAPHY

Mangroves are typical ecosystems of coastlines in the tropics, but may also reach into subtropical and mesic zones, e. g. on the Northern hemisphere, Baja California, Florida and South Carolina, the Red Sea, the Persian Gulf, Southern Japan and on the Southern hemisphere, Southern Brazil, South Africa, West and South Australia and New Zealand (see Figure 112 in Vareschi, 1980). The global area covered by mangroves is 140 000 km², which is only about 0.1 % of the total land surface of the earth. However, 60 – 75 % of all tropical coast-lines are occupied by mangroves which are highly important ecosystems, both ecologically and economically (Tomlinson, 1986, Hutchings and Saenger, 1987). Together with tropical rain-forests and coral-reefs mangroves are the most productive ecosystems on earth (Karsten, 1995).

In addition to coastlines of continents and islands, mangroves are also found around coral reefs. Mangroves can extend inland in river estuaries and at the coastlines in a

zonation up to an elevation of several tens of centimetres above sea level (Figure 1; Lin and Sternberg, 1992 a, b, 1993).

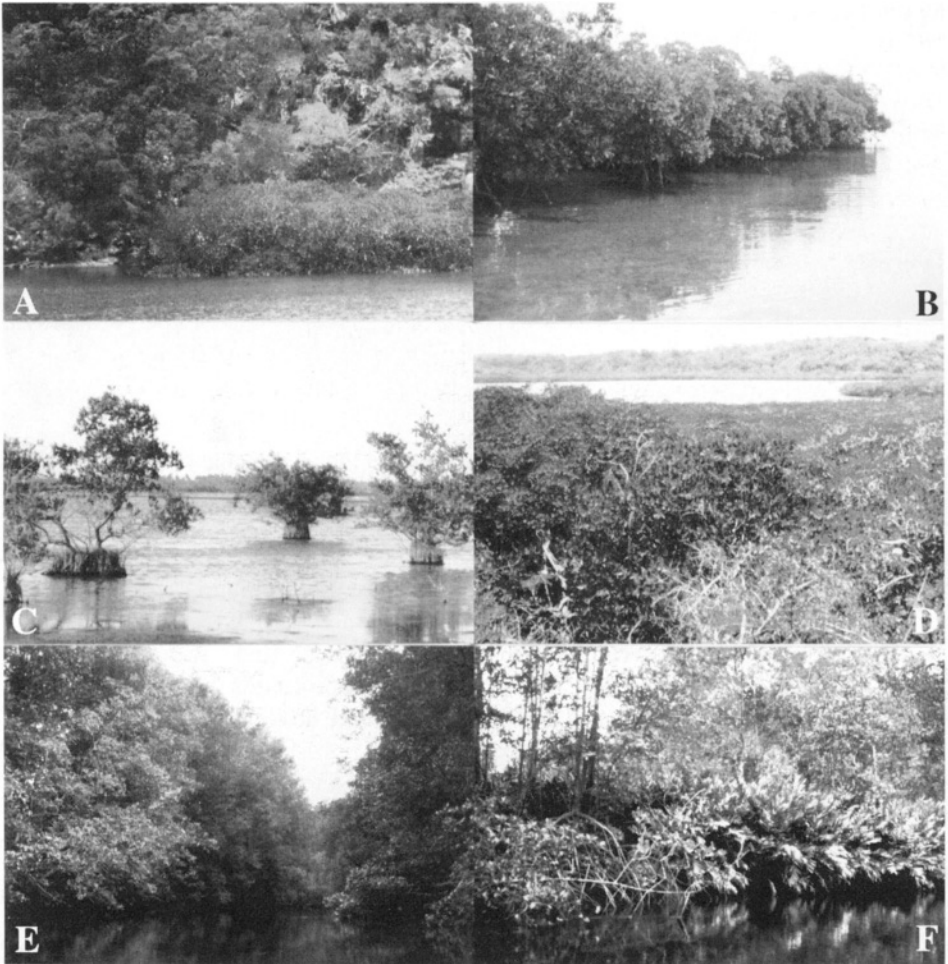


Figure 1. Mangrove habitats. A, B: Coastlines; Queensland, Australia (A); Fjdji (B). C, D: Lagoons; Chichiriviche, Venezuela, *Avicennia germinans* (C); Morrocoy, Venezuela (D). E, F: River mangroves, Rio Parrita, Costa Rica, with mangrove fern *Acrostichum aureum* (F). (see color version in the color section)

6.1.2 SITE CHARACTERISTICS

Site characteristics are given primarily by the tides, so that mangroves have also been considered as "tide-forests". However, the influence of the tides is modulated by the climate. At high tide, of course, salinity in the substrate of mangroves will be always determined by sea water. At low tide, however, salinity will be lower in humid climate, with rainfall frequently diluting and leaching salt, and higher in arid climate, with salt

concentrated by evaporation of water during the many hours, when the sea does not cover the substrate. Hence, in any case, mangrove sites are characterized by conditions of variable salinity, which may even change rhythmically.

Mangroves are characterized by their trees. In fact the term "mangrove" is derived from the tree genus *Rhizophora*, which is "mangle" in Spanish, and the English "grove". Trees in mangrove forests may become quite tall although often mangroves have scrub-like physiognomy. Lin and Sternberg (1992 a, b, 1993) found in Florida that *Rhizophora mangle* formed trees in the coastal fringe forest at levels around 24 cm above sea level, and a scrub formation is established at somewhat higher levels of 60 cm above sea level in transects, because the scrub sites at the higher levels are subjected to high salinities during the dry season. Although *Rh. mangle* can behave as a facultative halophyte and may benefit from fresh water in the rainy season, the rhythmic changes in salinity between sea water during high tide and fresh water during rainfall at low tide cause regular stress leading to a significant decrease in photosynthesis and productivity in the scrub sites as compared to the fringe forest sites with more constant salinity in the substrate.

6.1.3 COMMUNITIES

As mentioned above the physiognomy of mangroves is determined by the mangrove trees, and, indeed, mangroves are a special type of forest. The species diversity of mangrove trees is poor in the Americas (1 - 5 tree species) and also in Africa (4 species in W-Africa, 8 species in E-Africa and Madagascar) but quite respectable in Asia (about 25 species in India, and 30 species in SE-Asia) (see Figure 112 in Vareschi, 1980; and Popp, 1991). On the silty substrate of mangroves the undergrowth of vascular plants is usually poor (Ball, 1996) although the vigorous growth of a fern, the mangrove fern *Acrostichum aureum*, can be quite conspicuous. The gametophytes of this fern, however, are only resistant to mild salinity stress and can only survive the salinity of sea water for short periods, so that the fern remains restricted to the landward side of mangrove swamps (Li and Ong, 1998, Sun *et al.*, 1999). Most important, also in view of the productivity of mangroves, are the macro-algae, which often may also occur epiphytically on mangrove-tree roots, as well as microbe mats on the silty soil.

6.2 Mangrove trees

6.2.1 MORPHOLOGICAL CHARACTERISTICS

At the littoral habitats the wood of the trunks of mangrove trees needs to resist particularly strong winds as well as the pressure of tides. Most remarkable, however, are the root systems. In comparison to other tropical forest communities root biomass is greater in mangroves (Ball, 1996), which is important with respect to the problems of their substrate, e. g. salinity and inundation, as discussed below. Highly conspicuous is the diverse range of strangely shaped often bizarre root systems of mangrove trees

above the soil (Figure 2). They must have evolved to provide anchorage as well as aeration in the silty muddy soils and have, therefore, also been named pneumatophores. There are stilt roots, planks and buttresses and finger- or knee-like protrusions above ground which can serve gaseous exchange with the atmosphere or the sea water, depending on tidal level, while diffusion of gases is highly limited in the inundated soil.



Figure 2. Pneumatophores (A) *Rhizophora mangle*, (B) *Avicennia germinans*.
(see color version in the color section)

The root aeration via the pneumatophores is mediated by an interesting physical mechanism. The pneumatophores carry lenticels, i. e. openings in the bark which can be penetrated by gas but not by water. A pneumatophore aerenchyma may occupy as much as 70 % of total root volume (Curran, 1985). During high tide, root respiration lowers the O_2 -concentration in the intracellular spaces of the root aerenchyma to a hypoxia of as little as 10 % O_2 or even less. The CO_2 liberated concomitantly is released and dissolved in the sea water but O_2 cannot be reabsorbed readily from the water. Although photosynthetically active cells at the surface of the pneumatophores may contribute some O_2 for respiration (Aiga *et al.*, 1995), this causes considerable O_2 gradients along the roots and gas-pressure deficits in the aerenchyma of submerged roots to -1.7 kPa (Chiu and Chou, 1993; Skelton and Allaway, 1996; Youssef and Saenger, 1996; Löscher and Busch, 1999). At low tide, when the roots establish contact with the air again this leads air to being literally sucked into the root air spaces via the lenticels. Hypoxia is an additional stress to salinity in mangrove trees and in view of the energy costs of salinity tolerance (e. g. salt exclusion, K^+/Na^+ -selectivity, see below) such mechanisms for the control of hypoxia at the root level are quite important in addition to ventilation from the photosynthesizing shoot via the aerenchyma.

Some mangrove trees are viviparous. Seedlings grow out of flowers and fruits and remain for some time while on the mother plant. Once liberated the viviparous seedlings can establish themselves directly in the sediment at low tide or float in the sea water and are dispersed. The advantages of vivipary are not clear, however, since it is observed only in some mangrove tree taxa (e. g. *Rhizophora mangle*).

6.2.2 SALT AND OSMOTIC RELATIONS

6.2.2.1 *Salt exclusion, inclusion, dilution and excretion*

Salinity causes osmotic problems and in addition mainly also problems of ion toxicity of Cl^- and particularly Na^+ (Chapter 1). One way of escaping the latter is avoiding or at least restricting NaCl uptake at the level of the roots, i. e. salt exclusion. However, this strongly amplifies the osmotic problems. In order to maintain negative water potential gradients from the substrate to the various plant organs to allow water uptake and transport, salt excluders need to synthesise internal osmotica which binds a large amount of resources in terms of carbon skeletons, N and S (see below Figure 6) and consumes energy. The trade-off of complete or at least effective salt exclusion therefore, in most cases is negative, and it is no surprise that those glycophytes and crop species, which in adaptation to salinity realize salt exclusion, only achieve tolerance to rather mild substrate salinity (see also Chapter 14). Conversely, all *bona fide* halophytes and salinity tolerant plants are salt includers using the salt in their substratum as a readily available "cheap osmoticum".

However, salt exclusion *versus* salt inclusion never can be taken as an all or nothing distinction. All salt excluders to a certain extent allow salt uptake and all salt includers exert some control-over uptake at the root level. This is nicely exemplified surveying analytical data available for mangrove trees. Largely it appears that as true halophytes all mangrove trees are salt includers (Figure 3). Deviations of tissue contents of Na^+ and Cl^- from the average contents of these ions in sea water are small and mostly not larger than ca. ± 100 mM although in some cases higher deviations of ca. $+200$ mM and ca. -400 mM as compared to sea water have been reported (Figure 3). Accumulation almost equally affects both ions Na^+ and Cl^- with a small tendency of a larger Cl^- accumulation. While the average Cl^-/Na^+ -ratio in sea water is ca. 1.2, the Cl^-/Na^+ -ratios of the mangrove trees shown in Figure 3, average at 1.4 ± 0.2 ($\bar{x} \pm \text{SD}$, $n = 20$), with the exception of the three species where the salt content was ca. 400 mM less than that of sea water. Many mangrove tree species can grow in fresh water and behave as facultative halophytes. Like in other halophytes up to a certain level salinity stimulates growth, but high salinities inhibit growth to different extents in different mangrove species (Ball, 1996; Chapter 3). Thus, there is a range of compartments from moderate to high salt tolerance and obligate halophily (Ball, 1996).

Of course, tissue levels of salt are a product of dynamics of uptake and time and additional processes affecting the salt load of tissues, e. g. excretion as discussed below. Thus, data giving xylem levels of Na^+ and Cl^- are most interesting. They show that although allowing salt accumulation in their leaves close to sea water levels, mangrove trees are actually salt excluders, when we consider uptake as reflected by xylem sap concentrations. The levels of Na^+ and Cl^- in the xylem sap are 5 to more than 10 times less than those of sea water, in contrast to the levels in leaves (Figure 4). This underlines the necessity of using the terms salt exclusion and inclusion in careful balance with other observations.

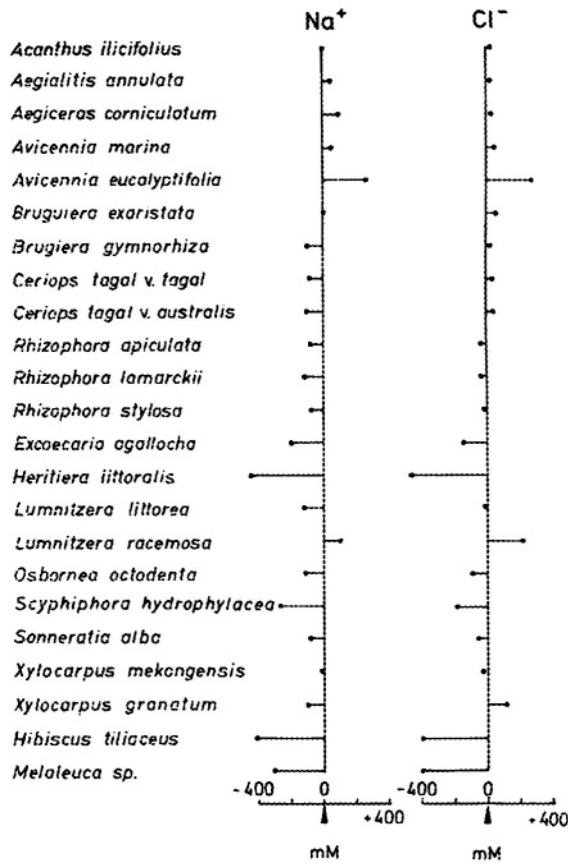


Figure 3. Na⁺ and Cl⁻ levels in tissues of various mangrove tree species collected from all over the world (Popp *et al.*, 1984) as indicated by their deviations from the average Na⁺ and Cl⁻ concentrations, respectively, in sea water.

Halophytes, including mangrove trees use two additional mechanisms for management of the salt load gradually building up during uptake in their tissues, viz. dilution and excretion.

Dilution is obtained by developing succulence, i. e. tissues with inflated cells and large vacuoles allowing storage of salt, where dilution occurs by osmotic uptake of water. Measurements with the mangrove tree *Laguncularia racemosa* have shown that when the actual Cl⁻ content in the leaves as given by the level of Cl⁻ per leaf area increases 10fold the Cl⁻ concentration in the leaf cells only doubles, because at the same time succulence increases about 4fold (Table 1). This also matches with observations on a *Sonneratia* species where the mature leaves had about 2.8 times the thickness of young leaves, and this was especially due to enlargement and vacuolization of the inner

mesophyll cells occupying 5.3 times the thickness on leaf cross sections, as leaves accumulated salt during maturation (Lear and Turner, 1977).

TABLE 1. Correlations between Cl^- -content, Cl^- -concentration and succulence in leaves of *Laguncularia racemosa*. (After data of Biebl and Kinzel, 1965; from Kinzel, 1982).

Cl^- -conc. (mmol m^{-2})	Cl^- -conc. (mM)	Succulence (kg m^{-2})
130	350	0.20
1220	700	0.85

Excretion is removal of salt from the tissue via salt glands (Chapter 3). This elimination of salt may even result in the formation of numerous salt crystals on leaf surfaces during dry sunny days (Figure 5) which may then dissolve hygroscopically at high relative humidity causing a salty rain underneath mangrove canopies at night and especially in the early morning. In the example of Figure 4 it is interesting to note that the mangrove species *Aegialitis annulata* having salt glands maintains no higher NaCl-concentrations in its leaves than the gland-less species *Rhizophora mucronata* although it allows more than 5 times the salt concentration in its xylem sap.

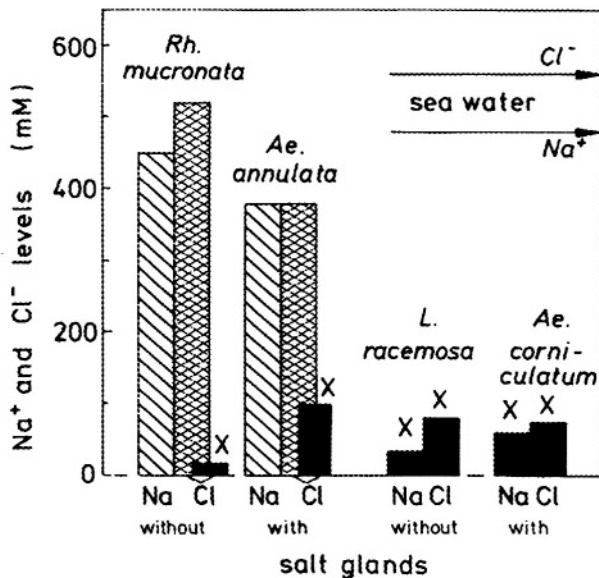


Figure 4. Na^+ and Cl^- levels in leaves (hatched columns) and xylem sap (X, black columns) of mangrove tree species analyzed in the field (*Rhizophora mucronata*, *Aegialitis annulata*: Atkinson *et al.*, 1967) and grown in sea water in a glass house (*Laguncularia racemosa*, *Aegiceras corniculatum*: Polania, 1990), respectively, without and with salt glands, respectively, in their leaves.

6.2.2.2 *Ion compartmentation and compatible solutes*

Membrane and enzyme machineries of halophytes are similarly sensitive to the toxic effects of Na^+ and Cl^- as those of glycophytes and this includes mangrove trees (Ball and Anderson, 1986; Sommer *et al.*, 1990). Hence, in addition to the mechanisms of salt load management described in the previous section, leaves of mangrove trees also need to protect their cytoplasm from the toxic ion effects. This is brought about by sequestration of NaCl in the vacuoles which, followed by osmotic water uptake, leads to the succulence ("salt succulence") described above. This, however, requires similar osmotic pressure, π , in the cytoplasm and vacuole because, by contrast to the plasma membrane, there is no elastic counter pressure at the tonoplast (i. e. a cell wall) counteracting π in the vacuole. Maintenance of identical π in cytoplasm and vacuole may not be achieved by loss of water from the cytoplasm to the vacuole because the functionally effective water structures at membranes and proteins must be maintained. This has led to the concept of "compatible solutes" (Chapter 9) which are osmotically active, and due to their molecular electron and charge distribution are similar enough to water-dipoles to be compatible with maintenance of cytoplasmic structures. Figure 6 presents a selection of such compounds actually found in mangrove trees and other halophytes. Metabolic costs of the plants for producing these substances remain affordable because the compatible solutes are only required in the relatively small cytoplasmic volume as compared to the much larger cell and tissue volume.

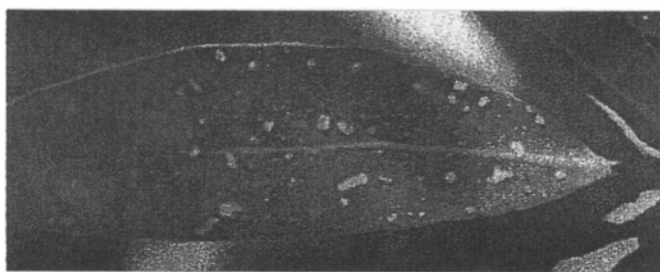


Figure 5. NaCl -crystals on a leaf of *Avicennia germinans*.

6.2.3 PHOTOSYNTHESIS

6.2.3.1 *CO_2 -exchange, stomatal conductance and water-use-efficiency*

CO_2 -exchange (J_{CO_2}), stomatal conductance for water vapour ($g_{\text{H}_2\text{O}}$) and water-use-efficiency (WUE) of mangrove trees in relation to the degree of substrate salinity have been studied by two groups, viz. Ball and Farquhar (1984 a, b) and Clough and Sim (1989). For an overview their data are averaged and summarized in Figures 7 to 9. Some simplification was also needed because units used by these authors were not always strictly comparable. Therefore, salinity is roughly indicated as that of 1/10, 1/2 and 1/1 of sea water. Nevertheless, the survey obtained gives a good indication of the general responses of mangrove trees.

Figure 7 shows that the effect of salinity on net CO_2 -uptake (J_{CO_2}) is not very pronounced up to 1/2-strength sea water, only *A. corniculatum* seems to be more sensitive than *A. marina* and the 19 species averaged by Clough and Sim (1989).

Full strength (1/1) sea water then reduces J_{CO_2} as stomatal conductance ($g_{\text{H}_2\text{O}}$) is also declining, but these effects are not dramatic. Internal CO_2 -partial pressure ($p_{\text{CO}_2}^i$)

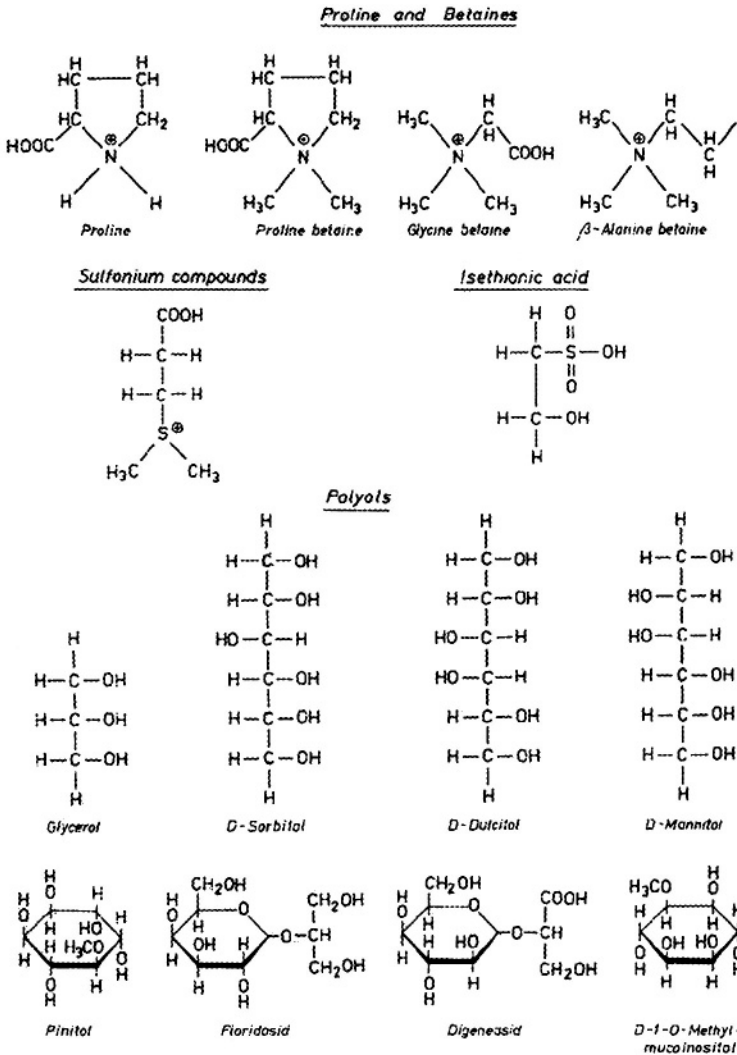


Figure 6. Compatible solutes in halophytes and in mangrove trees, fern and algae (Popp, 1984; Popp *et al.*, 1984, 1993; Richter *et al.*, 1990; Karsten, 1995; Karsten *et al.*, 1995 a).

remains between 150 and 250 Pa/MPa. The increase of $p_{CO_2}^i$ in *A. corniculatum*, while stomata partially closed (reduced g_{H_2O}) and J_{CO_2} strongly decreased at 1/1-strength sea water, may be due to some photoinhibition (see below section 2.3.2) in this case. In any of the cases shown in Figure 7 appreciable photosynthesis is still maintained at full strength sea water, as also shown by a comparison of the range of J_{CO_2} -rates in Figure 7 with rates of other ecological groups of plants, viz. glycophytic **C₃-plants** and even **C₄-plants** in the absence of salinity (Table 2).

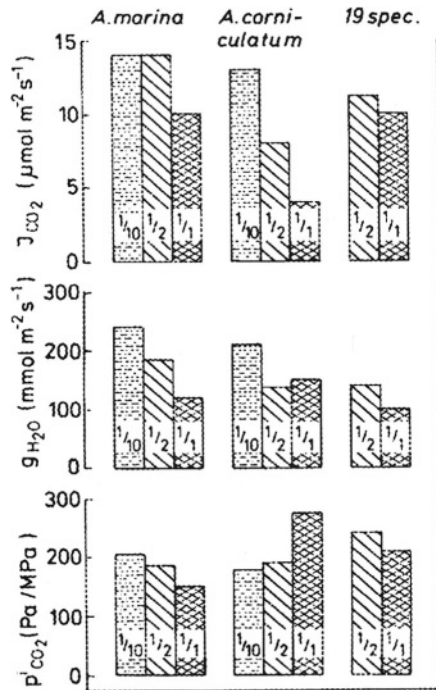


Figure 7. Net CO_2 -uptake (J_{CO_2}), stomatal conductance for water vapour (g_{H_2O}) and leaf internal CO_2 -partial pressure ($p_{CO_2}^i$) of the mangrove tree species *Avicennia marina* and *Avicennia corniculatum* (after Ball and Farquhar, 1984 a, b) and of averaged 19 different species studied in the field in Australia and Papua New Guinea (after Clough and Sim, 1989) at approximately 1/10-, 1/2- and 1/1-strength sea water as indicated in the columns.

With respect to salinity and osmotic stress of mangrove trees another derived parameter is of great interest, namely water-use-efficiency (WUE). Normally WUE-ratio is given by the simple relationship of carbon acquired and water lost concomitantly by evapotranspiration (J_{H_2O}), i. e.

$$\text{WUE-ratio} = J_{CO_2} / J_{H_2O} \quad (1)$$

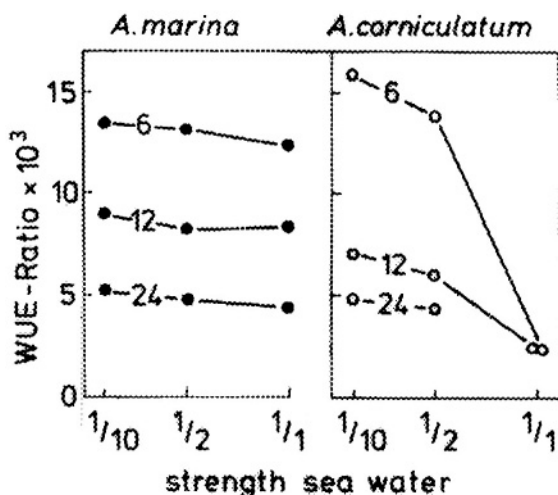


Figure 8. Water-use-efficiency ratios (Eq. (1)) of *Avicennia marina* and *Avicennia corniculatum* at approximately 1/10-, 1/2- and 1/1-strength sea water and different leaf-to-air water vapour pressure differences (VPD) as indicated by the numbers (in Pa/kPa) in the graphs. (After Ball and Farquhar, 1984 a.)

Data for mangrove trees from the work of Ball and Farquhar (1984 a) are plotted in Figure 8. WUE-ratio decreases with salinity only slightly in *A. marina* and more pronouncedly at 1/1-strength sea water in *A. corniculatum*. The graphs of Figure 8 also show that WUE-ratios not only are affected by substrate salinity but also by leaf-to-air water vapour pressure difference (VPD) with considerable decreases as VPD increases. However, Ball (1986) argues that notwithstanding these reductions of WUE-ratios the values obtained remain exceptionally high. Indeed, the WUE-ratios summarized for all conditions of salinity and VPD given in Figure 8 are higher than in glycophytic **C₃-plants** and even in **C₄-plants**, and most remarkably they are in the same range as obtained for the highly water saving nocturnal **CO₂-uptake** by crassulacean acid metabolism plants (Table 2).

For a more sophisticated analysis Clough and Sim (1989) have developed the concept of intrinsic water-use-efficiency. While stomatal control does, of course, affect J_{CO_2} and J_{H_2O} and hence WUE-ratio as given above (Eq. (1)), WUE-intrinsic is also based on the driving forces, i.e. the difference between external, $p_{CO_2}^a$, and internal, $p_{CO_2}^i$, **CO₂-partial pressure**, (driving force for net **CO₂-exchange** of the leaves) and VPD (driving force for transpiratory loss of water). It also incorporates the **CO₂-compensation point** of photosynthesis (Γ):

$$\text{WUE-intrinsic} = (p_{CO_2}^a - p_{CO_2}^i) / (p_{CO_2}^a - \Gamma) \quad (2)$$

where the relation to VPD is given by

$$\begin{aligned} p_{\text{CO}_2}^i &= p_{\text{CO}_2}^a - 1.6 \times J_{\text{CO}_2} \times \frac{\text{VPD}}{J_{\text{H}_2\text{O}}} = \\ &= p_{\text{CO}_2}^a - 1.6 \times \text{WUE-ratio} \times \text{VPD} = \\ &= p_{\text{CO}_2}^a - 1.6 \times \frac{J_{\text{CO}_2}}{g_{\text{H}_2\text{O}}} \end{aligned} \tag{3}$$

(The factor 1.6 in these equations is the ratio of diffusivities of H₂O-vapour to CO₂ in air.) The results show that WUE-intrinsic increases with increasing salinity and VPD (Figure 9) and suggest improved use of water as stomata partially close and VPD increases. Checking the data of Ball and Farquhar (1984 a; Figure 8) it can be seen that a similar conclusion might be obtained there as well. From Eqs. (2) and (3) it follows that

$$\text{WUE-intrinsic} = \frac{1.6 \times \text{WUE} - \text{ratio} \times \text{VPD}}{(p_{\text{CO}_2}^a - \Gamma)} \tag{4}$$

Assuming constant $p_{\text{CO}_2}^a$ and Γ (denominator in Eq. (4)) WUE-intrinsic for the values of Figure 8 in *A. marina* at all salinities would increase from 6 Pa/kPa to 24 Pa/kPa VPD by a factor of 1.3 to 1.5 and in *A. corniculatum* at 1/10- and 1/2-strength sea water by a factor of 1.25. At 1/1-strength sea water and VPD from 6 Pa/kPa to 12 Pa/kPa, the increase in WUE-intrinsic would be by a factor of 2. This is, of course, due to the fact that the increases in VPD were larger than the corresponding decreases in WUE-ratio.

Hence, the evaluation of both WUE-ratio and WUE-intrinsic shows that mangrove trees are well equipped for economic water use in their habitats which are characterized by

TABLE 2. Maximum rates of net-CO₂ uptake (J_{CO_2}) and water-use-efficiency (WUE) ratios for C₃-, C₄- and CAM-plants (Black, 1973) in comparison to the data of mangrove trees at various salinities (1/10-, 1/2- and 1/1-strength sea water) summarized in Figures 7 and 8.

	Maximum J_{CO_2} ($\mu\text{mol m}^{-2} \text{s}^{-1}$)	WUE-ratio $\times 10^3$
C ₃ -plants	10 - 25	0.6 - 1.3
Mangroves		
1/10 sea water	11 - 14	5 - 16
1/2 sea water	8 - 14	4 - 14
1/1 sea water	4 - 10	2 - 12
C ₄ -plants	25 - 50	1.7 - 2.4
CAM-plants		
Darkness	0.5 - 2.5	6 - 30
Light	7 - 8	1 - 4

high salinity and solar radiation leading to high VPD. With respect to the importance of VPD it is also necessary to mention leaf and air temperatures which in addition to atmospheric water vapour partial pressure are essential determinants of VPD. Reduced transpiration with increased WUE would reduce transpirational cooling. Leaf angle position towards solar radiation and morphological characteristics of leaves are additional attributes in optimization of these relations by mangrove trees (Ball, 1996).

Elevated atmospheric CO_2 -concentration ($p^a_{\text{CO}_2}$) modulates water use and carbon gain, and hence, one might expect that it affects salt tolerance of mangrove trees. However, a comparison of two *Rhizophora* species differing in salt tolerance, i. e. *Rh. apiculata* and *Rh. stylosa*, showed that elevated $p^a_{\text{CO}_2}$ is unlikely to increase salt tolerance. When relative growth rates were limited by salinity at the roots (i. e. at 350 mM as compared to 125 mM NaCl) elevated $p^a_{\text{CO}_2}$ from 340 to 700 ppm had little effect on relative growth rates. However, elevated $p^a_{\text{CO}_2}$ stimulated growth when this was limited by humidity at the leaves (i. e. at 43 % compared to 86 % relative air humidity) as given under conditions of aridity. Thus, elevated $p^a_{\text{CO}_2}$ in the future could modify competitive potentials of different mangrove trees along salinity x aridity gradients, although it is unlikely to allow mangrove trees to expand into areas with salinities much more extreme than currently tolerated (Ball *et al.*, 1997).

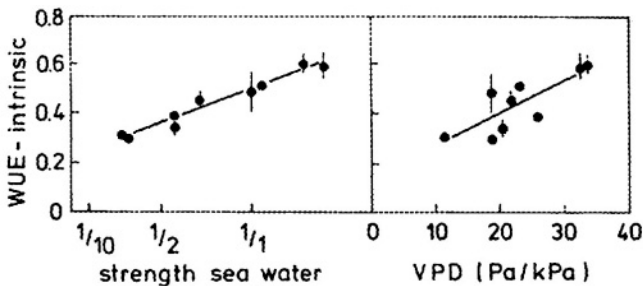


Figure 9. Intrinsic water-use-efficiencies (Eq. (2)) of averaged 19 different mangrove tree species studied in the field in Australia and Papua New Guinea in relation to approximate sea water strength and leaf-to-air water vapour pressure differences (VPD). (Clough and Sim, 1989.)

6.2.3.2 Xylem sap flow

The discussion of water relations of mangrove trees can not go without referring to xylem sap flow and the controversy that has arisen about the mechanism of water ascent in the xylem. Much of the pioneering work of Scholander and co-workers after the invention of the pressure chamber technique was devoted to mangrove trees, where xylem tensions of 3.8 to 5.2 MPa were recorded and accepted as sufficiently exceeding the osmotic pressure of sea water (2.5 MPa) to produce the driving force for sap ascent and a transpiration stream (Scholander, 1968; Scholander *et al.*, 1965, 1966). Conversely, Zimmermann *et al.* (1994 a) argue that the balancing pressure used in the pressure chamber technique overestimates xylem tension. Using staining techniques

they found viscous high-molecular-weight polymeric polysaccharide-mucilage in the xylem vessels of *Rhizophora mangle*. Such mucilage would tend to strongly support gas bubble formation, which would prevent stable xylem tensions larger than 0.1 MPa. The mucilage also would hinder a mass flow of water, and these authors quote observed sap flow rates in mangrove trees of $0.05 - 0.14 \text{ mm s}^{-1}$ which they consider to be low. They suggest that sap ascent is driven by gravity-independent streaming at gas/water interfaces (given by the gas bubbles) as well as a gradient of chemical activity of water established by the - potentially highly hygroscopic - mucilage attracting and holding water. Conversely, Becker *et al.* (1997) note that xylem clogging by mucilage in mangroves at least can not be generalized, and they report sap flow data in mangrove trees which they think are not that low ($0.09 - 0.16 \text{ mm s}^{-1}$) and in fact compare well to those of other tropical trees. They found appreciable stomatal conductances in well illuminated leaves of *Avicennia cf. alba* of $400 \text{ mmol m}^{-2} \text{ s}^{-1}$ (i. e. much higher than the average values compiled in Figure 7) and measured midday leaf water potentials of -3 MPa in *A. cf. alba* and *Rhizophora apiculata*. Thus, in their view, the cohesion-tension theory of sap ascent remains applicable to mangrove trees.

This is not the place for getting involved in detail in the controversy on the cohesion-tension theory (Zimmermann *et al.*, 1994 b; Tyree, 1997; Lösch, 1998; Wei *et al.*, 1999 a, b; Chapter 12). With respect to mangrove trees it is interesting to note that with their special ecophysiological problems they play a part in this game. It may be appropriate, however, to conclude with Becker *et al.* (1997) that "like plants of other vegetation types, mangrove species will probably exhibit a range of transpirational behaviours in response to their saline habitat once they have been more fully investigated". Hydraulic architecture plays a large role in such comparisons (Ball, 1996). Sobrado (2000) found that the hydraulic systems of three mangrove tree species *Avicennia germinans*, *Laguncularia racemosa* and *Rhizophora mangle* were comparable to the lowest end of the range reported for tropical trees. Instantaneous water-use-efficiency ratios of the leaves of the three mangrove trees declined in the sequence given. Conversely, *L. racemosa* and *A. germinans* showed less efficient water transport at the shoot level. These hydraulic properties may be important for the ecophysiological comportment and niche occupation in the habitat.

6.2.3.3 Photoinhibition and photoprotection

Photoinhibition is most readily assessed by measuring chlorophyll fluorescence, where potential quantum yield of photosystem II of dark adapted leaves (F_v/F_m ; with F_m maximum fluorescence and F_v variable fluorescence of dark adapted leaves) of $\cong 0.83$ indicates healthy leaves and lower F_v/F_m ratios indicate more or less strongly photoinhibited leaves (Björkman and Demmig, 1987). Cheeseman *et al.* (1997) obtained dawn values of ~ 0.8 for *Rhizophora stylosa* under extremely challenging field conditions. Sobrado (1999) studied *Avicennia germinans* during rainy and dry season at a high salinity site (30 – 50 ‰ in the wet season, 60 ‰ in the dry season) and a low salinity site (5 - 15 ‰ and 40 ‰ in the wet and dry season, respectively; where 30 - 35 ‰ correspond to 0.52 - 0.55 M NaCl, i. e. the salinity of sea water). She obtained predawn F_v/F_m values of ~ 0.75 under all conditions, i.e. only mild Photoinhibition, which was not reversed over night. Similar predawn values of F_v/F_m

were also measured by Sobrado and Ball (1999) with *Avicennia marina* under 1/1 sea water salinity and hyper-salinity of 2/1 sea water. These authors conclude that this could still be reconciled with dissipation of excess light energy via the xanthophyll cycle (see below) and that there was no evidence for chronic depression of F_v/F_m due to salinity. Thus, mangrove tree chloroplasts must be well protected against chronic photoinhibition and photodestruction. Protection may occur via the Mehler reaction reducing oxygen to water, and as stress increases by O_2^- scavenging by ascorbate or enzymatic detoxification by superoxide dismutases (SOD). Indeed ascorbate levels were found sufficient in *Rhizophora stylosa*, and total SOD activities in *Rh. stylosa* and *Rhizophora mangle* were 38 times and 6 times, respectively, those of *Pisum sativum* used for comparison (Cheeseman *et al.*, 1997).

However, mangrove trees certainly are subject of acute photoinhibition during high insolation. While Cheeseman (1994) did not observe photoinhibition in *Rhizophora mangle* under water-stress in the greenhouse as well as in sun exposed *Bruguiera parviflora* in the field (Cheeseman *et al.*, 1991), Björkman *et al.* (1988) reported a large decrease of F_v/F_m for various mangrove species at high solar radiation in the field and Sobrado (1999) detected F_v/F_m of 0.45 to 0.55 at midday in the different seasons and sites mentioned above.

Acute photoinhibition not reversible after short periods of darkening has protective functions since excess photosynthetic excitation energy is dissipated in a harmless way, mainly in the form of heat. Another mechanism is destruction of proteins of the PS II-light-harvesting complex, e.g. the D1 protein, which due to its high turnover is readily repaired within several hours (Thiele *et al.*, 1998).

Heat dissipation is mediated by the xanthophyll cycle, via zeaxanthin and its epoxidation products antheraxanthin and violaxanthin (Demmig-Adams, 1990). Lovelock and Clough (1992) did find that the depression of F_v/F_m in mangrove-tree leaves at midday was in fact correlated with the concentration of zeaxanthin per unit leaf area. Sobrado and Ball (1999) compared *A. marina* at 1/1- and 2/1-strength sea water salinity. The hypersaline condition reduced net photosynthetic CO_2 -uptake (J_{CO_2}) from 7.6 to 4.3 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and stomatal conductance for water vapour (g_{H_2O}) from 123 to 53 $\text{mol m}^{-2} \text{s}^{-1}$. Under these conditions, and despite the much reduced CO_2 -assimilation under the hypersaline regime, xanthophyll pool sizes and epoxidation states as well as non-photochemical energy dissipation (i.e. not connected to CO_2 -assimilation) were similar in both sets of plants, i.e. at 1/1- and 2/1-sea water salinity. These results also imply that in the hypersaline regime more excitation energy must have been dissipated via photorespiration, although this remains necessary to be shown in more detail. Other alternative electron sinks may have also been involved. In any case, under the natural conditions of this study there was no increased xanthophyll-cycle dependent photoprotection or non-photochemical dissipation of excess excitation energy despite the 43 % decrease in CO_2 -assimilation rate with the doubling of the salinity level.

Another important observation is that polyols, which are the dominating compatible solutes in mangroves (Figure 6; section 2.2.2), may not only act as osmolytes and

protective substances for water structures of enzyme and membrane systems, including chloroplasts, but also may be very effective radical-scavengers (Orthen *et al.*, 1994). Li and Ong (1998) and Sun *et al.* (1999) studied this in gametophytes of the mangrove fern *Acrostichum aureum*. Polyol concentration was strongly correlated with Na^+ accumulation in the gametophyte tissue as determined by substrate salinity, and this was associated with a considerable increase of F_v/F_m in light treated (photosynthetically active photon fluorescence density of $400 \mu\text{mol m}^{-2} \text{s}^{-1}$) gametophytes after 30 min dark adaptation (Figure 10).

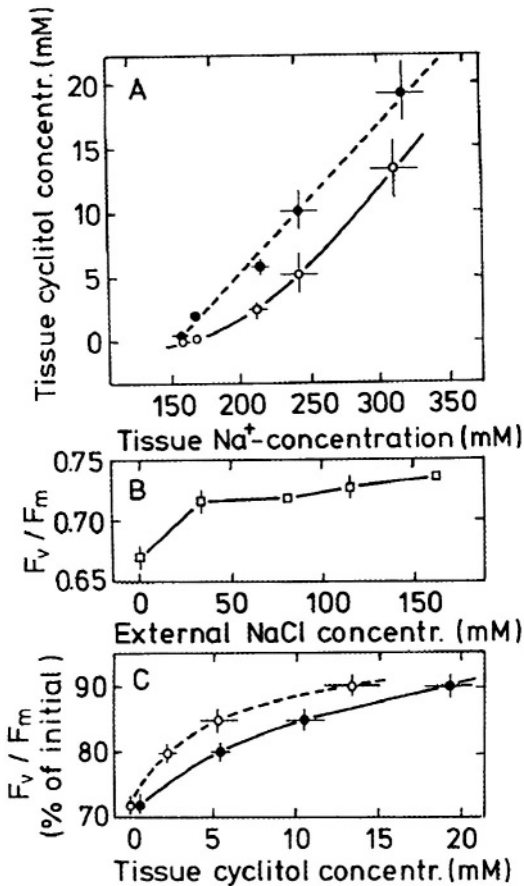


Figure 10. Salinity dependent accumulation of polyols and correlated increase of potential quantum yield of photosystem II (F_v/F_m) after 30 min of dark adaptation in gametophytes of the mangrove fern *Acrostichum aureum*. (Closed circles total polyols, open circles D-pinitol; errors are SE.) (A): Correlation of polyol and Na^+ concentration in the tissue. (B) and (C): Gametophytes grown under the external salinities indicated by the abscissa in (B) and then transferred to 340 mM NaCl for 2 days. Hardening by growth at increased salinity (A) is demonstrated by increased F_v/F_m (B) and this is correlated to tissue polyol concentration (C). (Sun *et al.*, 1999.)

6.3 Aquatic communities

6.3.1 MACROALGAE OF MANGROVES

Macroalgae in mangroves grow between the roots of mangrove trees but mainly epiphytically on the pneumatophores and trunks of the trees (Post, 1963). Hence, they are subject to the same stress conditions and even more so than the woody mangrove plants, i.e. changing salinity, desiccation at low tide, irradiance and temperature. Species diversity is mainly given by red algae of the genera *Bostrychia*, *Caloglossa* and *Stictosiphonia*, although brown algae may also occur, e.g. mats of *Hormosira banksii* in SE-Australia (Karsten, 1995). About 15 - 20 % of the total biomass of mangroves is represented by these macroalgae (Karsten, 1995).

In adaptation to their very stressful habitat mangrove algae show a broad salinity tolerance between 0.2 x and 2 x strength sea water (Karsten and West, 1993). Some mangrove algae are also desiccation tolerant (Biebl, 1962), e.g. *Stictosiphonia arbuscula* can loose up to 95 % of its tissue water and recover its metabolism again within several hours when wetted (Karsten, 1995).

Osmolytes and compatible solutes (see above section 2.2.2) are accumulated by the red algae as a function of salinity (Karsten *et al.*, 1995 a), and their protective function has been tested with enzyme extracts from various mangrove algae (Karsten *et al.*, 1996). Compatible solutes in red algae of mangroves show a rather high chemical diversity (Figure 6), they include floridoside, digeneaside, D-sorbitol, D-dulcitol, D-mannitol, isethionic acid (Karsten, 1995, Karsten *et al.*, 1995 a). Mannitol is a rare sugar alcohol among red algae, and *Caloglossa* seems to be the only genus where mannitol is synthesized, and mannitol metabolism has been studied (Karsten *et al.*, 1997 a, b). Different compatible solute spectra have been found in mangrove algae from different geographic provenance, e.g. in *Bostrychia tenuissima* from Australia sorbitol plus dulcitol and sorbitol plus digeneaside, respectively, and this was controlled genetically (Karsten *et al.*, 1995 b). In the mangrove fern *Acrostichum aureum* different osmolytes occur in the gametophyte and in the sporophyte with D-pinitol in the former and D-1-O-methyl-muco-inositol in the latter (Sun *et al.*, 1999).

Isethionic acid is an interesting rare compound which is formed by direct reaction of ethylene with SO_3 and subsequent hydrolysis of the ethionic acid ($\text{HO}_3\text{S} - \text{CH}_2 - \text{CH}_2 - \text{OSO}_3\text{H}$). The phytohormone ethylene is often produced in plants in response to inundation and hypoxia (Abeles *et al.*, 1992). The reducing substrate of the silty soil of mangroves is also producing H_2S (see section 6.3.2), which would then need to be oxidized to SO_3 .

Another important factor for growth of mangrove algae is irradiance. While the canopy of mangrove trees may receive photosynthetic photon flux densities (PPFD) up to $2500 \mu\text{mol m}^{-2} \text{s}^{-1}$, the algae may not obtain more than 60 to $100 \mu\text{mol m}^{-2} \text{s}^{-1}$. This is not only due to shading by the trees but also to turbid water with organic materials and debris (Karsten, 1995). Thus, mangrove algae are extreme shade plants with low light compensation points of photosynthesis and low saturating PPFD (Karsten and West, 1993, Karsten *et al.*, 1994). *Bostrychia simpliciuscula* and species of *Caloglossa* may

still show good positive relative growth rates at the very low PPFD of $2.5 \mu\text{mol m}^{-2} \text{s}^{-1}$. A small decline of growth at increasing PPFD is often observed, and then growth rates increase up to $40 - 60 \mu\text{mol m}^{-2} \text{s}^{-1}$ PPFD, while higher irradiances become inhibitory (Figure 11; Karsten and West, 1993, Karsten *et al.*, 1994).

6.3.2 MICROBIAL MATS

Although Alongi (1994) concluded that due to the light limitation on the floor of mangrove forests photosynthesis by benthic microalgae only makes a minor contribution to primary productivity, microbial mats generally appear to be a very essential part of mangrove ecosystems. Karsten (1995) estimates that they account for 5 – 20 % of the total mangrove productivity. They are largely composed of diatoms, cyanobacteria, sulfur bacteria, purple sulfur bacteria and sulfate reducing bacteria (Table 3). Their thickness as given in Table 3 is about 10 to 12 mm (Karsten, 1995), but can also be as mighty as 80 – 120 mm (Hussain and Khoja, 1993). The main stress factors, as for the other mangrove communities subject to the tidal effects, are large, varying amplitudes of salinity, irradiance and desiccation.

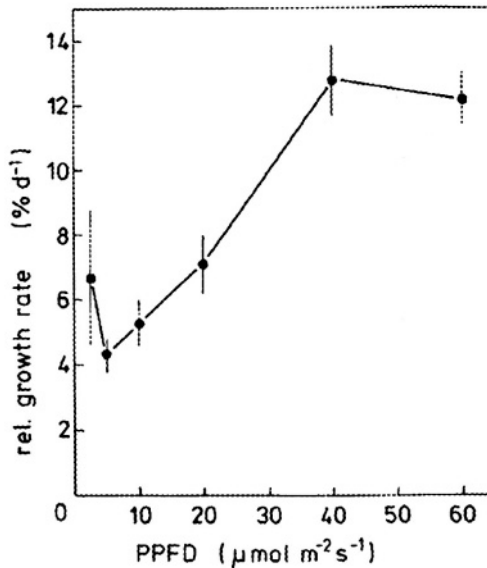


Figure 11. Relative growth rate (increasing length of laterals during 4 - 6 days) of *Caloglossa* species in relation to photosynthetic photon flux density (PPFD). Data are averages of the measurements of 6 different species presented by Karsten and West (1993) \pm standard errors.

Cyanobacteria use glycosylglycerol as osmolyte (Karsten, 1996). From events of high irradiance with a large component of UV-B they protect themselves forming ultraviolet absorbing substances such as scytonemin, pterins and myco-sporine-like amino acid

compounds (Karsten *et al.*, 1998). Often the upper layer of microbial mats in mangroves is formed by *Lyngbya* cf. *aestuarii* which particularly accumulates scytonemin and then also provides a sunscreen for the entire benthic community with more shade adapted species underneath (Karsten *et al.*, 1998).

TABLE 3. Layers of microbes in microbial mats of mangroves (after Karsten, 1995).

thickness from top (mm)	Organisms
0 - 2	fine sand plus diatoms
2 - 4	cyanobacteria
4 - 6	sulfur bacteria
6 - 8	purple sulfur bacteria
8 - 11	sulfate reducing bacteria
11 -	Sand

The microbial mats play a very important role in the biogeochemistry of the sediments forming the mangrove soil. Although crabs may enhance breakdown of litter > 75 times as much as microbes (Robertson and Daniel, 1989) microbial breakdown remains important. Soil microbes also determine the redox potential in the sediment (Boto, 1982; Valiela, 1984; see Table 3) which affects nutrient availability, especially phosphorus (Ball, 1996). Dinitrogen fixing bacteria and cyanobacteria make important contributions to the N-input into mangrove ecosystems, and thus, contribute to their high productivity (Zuberer and Silver, 1978, 1979; Sengupta and Chaudhuri, 1991).

6.4 Conclusion

Mangroves are among the endangered ecosystems on earth. They have been frequently considered to be useless and are disappearing rapidly. They are very unique, and with their characteristic beauty they are among the outstanding natural heritages we have. However, it must be noted that they can also be very directly important for us (Cormier-Salem, 2000). They are pioneer communities at the interface between sea and land and stabilize coastlines. They provide valuable timber. They serve as nurseries for breeding of marine life, for fish and crabs of coral reefs, and provide the economical basis of coastal fisheries. They have already been used for establishing ponds for the culture of fish and prawns. If we take the productivity of macroalgae as 15 - 20 % and that of microbial mats as 5 - 20 %, the productivity of trees in this highly productive ecosystem would be 80 to 60 %, which underlines the diversity in the system.

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C. MECHANISMS

Mechanisms of salinity interactions reflect the organizational levels and the levels of different scaling of membranes, cells, tissues, organs and the whole plant. Thus, we observe ultrastructural effects of salinity (Chapter 7). Cellular ion compartmentation (Chapter 8), directly leads into consideration of osmoregulation and the concept of compatible solutes (Chapter 9). Interactions between various mineral ions, organic compounds and water molecules are thus involved in mechanisms of salinity relations, and mineral nutrition at large (Chapter 8). Specific effects of salinity, such as sodium-calcium interactions (Chapter 10) and interference with nitrogen-nutrition (Chapter 11), are important for the overall behavior of the plants under salinity stress. Water relations are highly affected by this whole array of salinity-stress related responses, where osmotic effects, specific salt or ion effects and water availability and supply to membranes, proteins, cells, tissues and organs are often not easily separable from each other. Hence, it is not astonishing that the salinity literature as covered in these chapters often rather heavily draws also on studies of drought-stress physiology. Clearly, this is also pertinent very much to considerations of water flow in the whole plant *via* the xylem conduits (Chapter 12). Functioning of the whole network of interactions elucidated here allows plant growth and development under salinity, but this requires complex signaling in which phytohormones may play a significant role (Chapters 13,14).

CHAPTER 7

ULTRASTRUCTURAL EFFECTS OF SALINITY IN HIGHER PLANTS

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Abstract

Salinity leads to structural and ultrastructural effects, particularly in salt-sensitive species. Some of them are indicative of the onset of injury, for example the aggregation of chloroplasts accompanied by a swelling in the granal and fret compartments or the complete distortion of chloroplastic grana and thylakoid structures. Others are associated with metabolic acclimation to salinity stress. For instance increased density of mitochondria, enhanced ATPase particle frequencies in membranes may be related to enhanced energy demand at moderate salinity. Salinity-induced ultrastructural changes, such as the build up of transfer cells and many small vesicles, may be a sign of extensive exchange of substances across membranes. Several examples of structural adjustment of halophytic and glycophytic plant species will be presented in this chapter.

7.1 Introduction

Salinity is an environmental stress that limits the growth and development of salt sensitive plants (Greenway and Munns, 1980) but promotes the growth of halophytes even at relatively high levels of NaCl. Usually there is a threshold concentration of salt above which both groups begin to show signs of anatomical and morphological changes, growth inhibition, leaf discoloration and loss of dry weight. However, most halophytes show also symptoms of deficiency if the NaCl concentration in the substrate remains below a distinct level.

Thus, besides chemical and analytical procedures, current research on salinity responses must include methods using light and electron microscopy to find incipient symptoms of disorders with a view to improving the diagnosis of conventional crops

and cash crop halophytes (Lieth and Al Masoom, 1993; Huiskes, 1995; Koyro and Huchzermeyer, 1999). Physiological information about impairment of cell metabolism is where possible combined directly with studies of structural changes (Hecht-Buchholz and Marschner, 1970; Hecht-Buchholz *et al.*, 1974; Hecht-Buchholz, 1983; Huang and Van Steveninck, 1990; Koyro, 1997). Additionally, adaptive mechanisms under conditions of NaCl-stress are connected to the structural behaviour of a cell or tissue in order to explain tolerance and efficiency mechanisms. Investigations have been carried out with light and electron microscopy to find differences in cell structure and anatomy, which could explain differences in tolerance of plant species or even of genotypes towards mineral excess or deficiency.

Special attention is drawn to cytological changes in association with the physiological adaptation of plant species to excessive NaCl stress. The resistance of halophytes to salts is not a consequence of salt-resistant metabolic pathways. Instead, other mechanisms come into play to avoid salt injury. Several examples of structural adjustment to NaCl-salinity in which particular reaction patterns enable the plant to cope with the prevailing conditions in the edaphic environment will be shown in this chapter.

7.2 Structural effects of NaCl-salinity in epidermal and outer cortex cells of the root

7.2.1 STRUCTURAL PROPERTIES OF THE CELL WALL

The epidermis cells play a key function in the uptake of minerals into the symplast of the plant. Direct exposure of the root cortex to NaCl led to several structural changes in these cells, a fact which may explain differences in salt tolerance (Koyro, 1997).

The root tip acts as a finely tuned sensor for different kinds of stress (Roth and Bergmann, 1988; Colmer *et al.*, 1994). Salt and drought stress were found to be associated in the root tips of several species such as *Sorghum bicolor* x *S. sudanensis* with a shortening of the growth region (Wilson and Robards, 1978; Clarkson *et al.*, 1987; Bernstein *et al.*, 1993). The elongation zone of *Sorghum* leaves was also shorter in salinity despite the fact that the duration of elongation growth associated with a cell during its displacement from the leaf base was longer in salinized leaves than in the control (Bernstein *et al.*, 1993).

Root cells exhibit complete regulation of single-cell turgor when osmotically stressed with low ($40 \text{ mol} \cdot \text{m}^{-3} \text{ NaCl}$) or high (seawater) NaCl salinity (Pritchard *et al.*, 1991; Munoz *et al.*, 1996). This result suggests an effect of NaCl on the yield threshold through changes in wall extensibility. Changes in wall rigidity can be explained by salt-induced changes in the cell wall of the epidermis cells as shown by Munoz *et al.* (1996) and Koyro (1997):

- (a) Salinity induced the storage of the β 1,3D-glucan, callose, in the cell walls of the outer cortex cells in *Sorghum*.

- (b) Freeze etching replicas present a typical secondary cell wall in the epidermis cells of controls whereas salt-treated cells present mainly primary cell walls (Figure 1).
- (c) The effect of pure seawater on outer cortex cells of *Prosopis alba* was to increase the presence of turgid cells with thicker walls.

Binzel *et al.* (1987) have shown that alterations in the physical properties of the cell wall take place in cells adapted to NaCl stress. Carbon can be partitioned for osmotic adjustment at the expense of cell wall synthesis. There is considerable data supporting the hypothesis that the hydrolysis of glucans permits wall expansion. It is possible that the deposition of a continuous layer of callose on the inner wall surface hinders the expansion of the root cells (Currier, 1957; Koyro, 1997).

The arrangement of microfibrils in parallel along the longitudinal cell walls of rhizodermal cells, as shown for controls of *Sorghum*, supports the enlargement of cells in one (i.e. longitudinal) direction (Lüttge *et al.*, 1999). There seems to be a connection between the arrangement of the cells and intracellular ABA-concentrations (Schnepf and Reinhard, 1997). In corresponding cells of salt-treated roots, cells show no preference for cell enlargement. The arrangement of microfibrils in the longitudinal cell walls in a closely meshed network supports uniform cell extension (Koyro, 1997).

7.2.2 INTRAMEMBRANEOUS PARTICLES

Comparative investigations have been conducted with isolated vacuoles of leaves of *Mesembryanthemum crystallinum* (Klink *et al.*, 1990) and with intact root tissues of several plants such as drought-sensitive *Sorghum* and salt tolerant *Spartina* plants grown under steady-state conditions with or without $40 \text{ mol} \cdot \text{m}^{-3}$ NaCl (Wolfe, 1979, 1987; Robards and Clarkson, 1984; Koyro *et al.*, 1993; Pei and Zhang, 1995). Salt treatment caused an increase of epidermal IMP (intramembraneous particle) frequencies in the plasmalemma and tonoplast of rhizodermal cells of *Sorghum* and *Spartina*. This is interpreted by the authors in terms of biochemical and/or physiological differences, especially those of membrane-bound enzymes (such as ATPases). This supposition was confirmed by a salt-induced increase in ATPase-activity (F-, T- and P-ATPase) per μg protein. The authors conclude from this that selective properties of the rhizodermal membranes such as a preferential uptake of K^+ over Na^+ ($\text{S}_{\text{K,Na}}$) or sucrose transport increase owing to increases in IMP.

Spartina roots possess a low $\text{S}_{\text{K,Na}}$ at the plasmalemma of rhizodermal cells and adapt osmotically mainly with NaCl, thereby causing low energy costs (Yeo, 1983). *Sorghum* roots accumulate mainly organic compounds for this adaptation (Weimberg *et al.*, 1982, 1984). The high energy demand required for osmotic adaptation, along with the high K-over-Na-selectivity, provides a possible explanation for the reduction in growth.

7.2.3 VESICLES, MITOCHONDRIA, ENDOPLASMIC RETICULUM AND POLYSOMES

NaCl salinity (between 25 and $50 \text{ mol} \cdot \text{m}^{-3}$) often increases the amount of vesicles and mitochondria in root cells (Hecht-Buchholz *et al.*, 1971, 1974; Walker and Taiz, 1988;

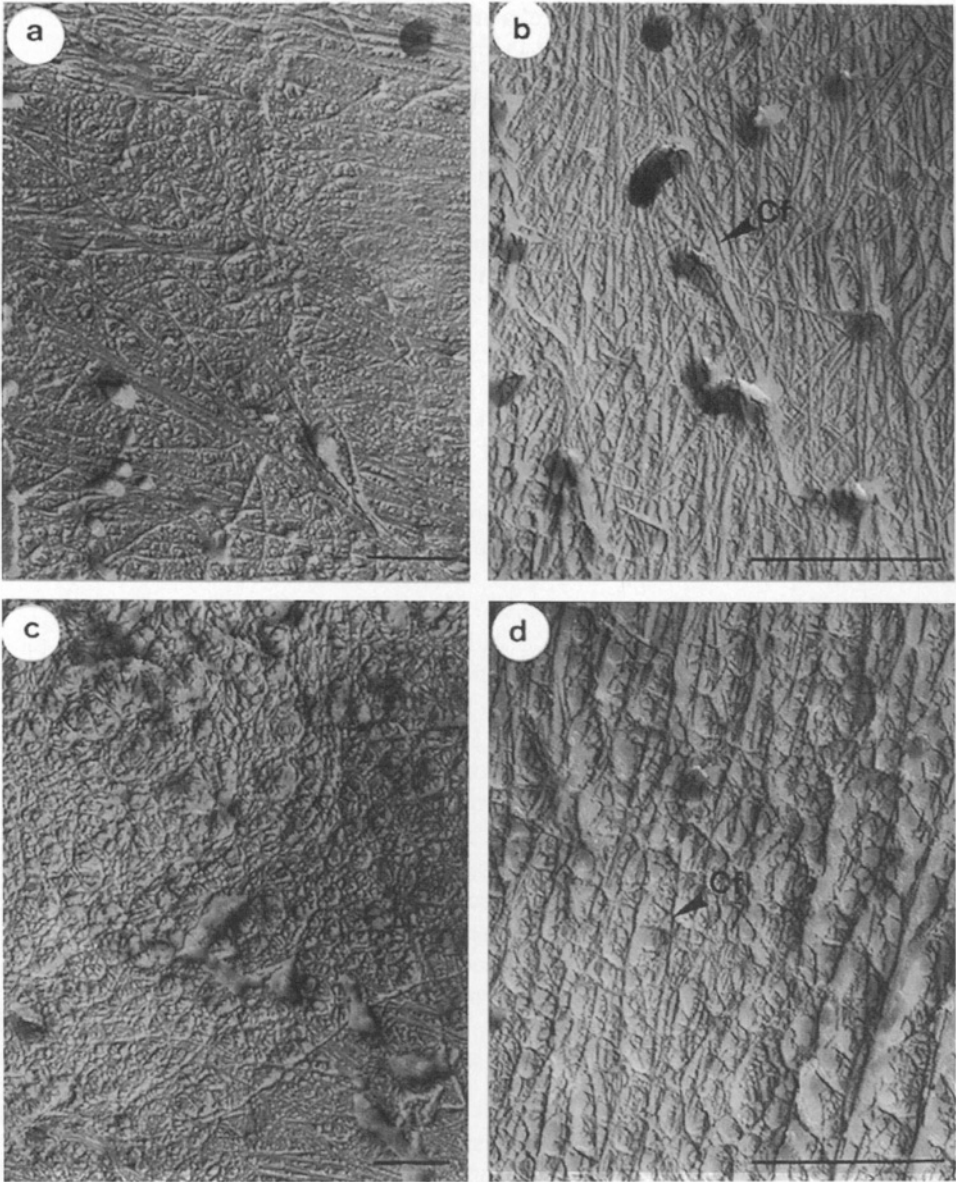


Figure 1: Electron micrographs of freeze-fracture replicas from epidermal cells of *Sorghum* roots tips. (a) and (b): In control roots, the microfibrils (cf) are arranged mainly in a parallel array. (c) and (d): In corresponding cells of the salt-treated roots (40 mol * m⁻³ NaCl), the microfibrils form a close-meshed network. Scale bar represents 100 nm.

Huang and Van Steveninck, 1990; Koyro, 1997). Salinity may increase the respiration rate of the roots, which have a higher carbohydrate requirement for maintenance respiration in saline substrates (Schwarz and Gale, 1981).

In addition, an increased number of microbodies, aggregation of ribosomes with polysomes, and also accumulation of ER (endoplasmic reticulum) parallel to the walls could be observed in maize root tips. The accumulation of microbodies and endoplasmic reticulum in sodium-stressed plants is indicative of increased detoxification processes in senescent and stressed tissue (Bergfeld and Falk, 1968; Berger and Schnepf, 1970; Koyro, 1997). The form of polysomes indicated the presence of active protein synthesis. However, none of these factors alone was indicative of tolerance mechanisms.

7.2.4 TRANSFER CELLS IN THE EPIDERMIS

It was possible to accelerate vacuolation in the apical region of barley and *Sorghum* root tips by treatment with $50 \text{ mol} \cdot \text{m}^{-3}$ and $40 \text{ mol} \cdot \text{m}^{-3}$ NaCl, respectively (Huang and Van Steveninck, 1990; Figure 2). Cortex-, epidermis- and endodermis cells of *Sorghum* roots of controls show e.g. $400 \mu\text{m}$ behind the root tips a beginning (or no) vacuolation. Cortex cells of plants cultured with $40 \text{ mol} \cdot \text{m}^{-3}$ NaCl present in the same distance to the tip already a big central vacuole, the epidermis and endodermis cells a beginning vacuolation. This NaCl-effect can be explained by a decrease of the longitudinal expansion of cells. Additionally, the build-up of transfer cells has been shown for epidermal cells of salt-treated plants (Kramer *et al.*, 1978; Kramer, 1983; Winter, 1988; Koyro, 1989).

There appears to be a correlation between the build up of transfer cells and many small vesicles with an extensive exchange of substances across the plasmalemma and/or tonoplast membranes. It is considered that these changes are connected with K^+/Na^+ -discrimination and with increases in the exchange capacity between compartments. The increase in vesicular surface and the build up of transfer cells indicates, particularly in epidermal cells, a higher and more selective uptake and storage of substances. In maize cultivar differences in the degree of Na^+ exclusion seem to be related to differences in passive Na^+ permeability of the root cell membranes (Schubert and Lächli, 1990).

7.2.5 ELECTRON OPTICAL CONTRAST

Apart from indicating changes in the quantity of organelles, it was possible to demonstrate a salt-induced increase in electron optical contrast in the cytosol and in organelles such as the mitochondria in halophytic and glycophytic species (Nir *et al.*, 1966; Huang and Van Steveninck, 1990; Koyro, 1997). It is believed that there is a connection between the decrease in Mg, K and Ca-concentrations in the cytoplasm, the decrease in electron optical contrast, and the inhibition of essential enzymes (Koyro, 1997; H.-W. Koyro unpubl. results). Cultivars with higher salt tolerance have a higher capacity to maintain lower Na^+ concentrations in the cytoplasm (Hajibagheri *et al.*, 1989).

Ca^{2+} seems to be beneficial in maintaining a high pH-gradient between cytoplasm and vacuole, and thus, sustains the putative driving force for Na^+ transport from the cytoplasm into the vacuole via a Na^+/H^+ antiport as well as levels of cytoplasmic K^+ , cytoplasmic and vacuolar phosphate (Colmer *et al.*, 1994; Tazawa *et al.*, 1995). Ca^{2+} can alleviate the inhibitory effect of NaCl on root growth by maintaining plasmamembrane selectivity of K^+ over Na^+ (Zhong and Läuchli, 1994). Salt-induced changes in the ultrastructure and element composition of epidermal root cells can be explained in terms of a Ca^{2+} deficiency leading to loss of control over cytoplasmic homeostasis. However, it was shown that salt-induced changes in electron optical contrast can be induced artificially if NaCl is added to the fixation solutions of control plants (cultured without additional NaCl; Hwang and Chen, 1997).

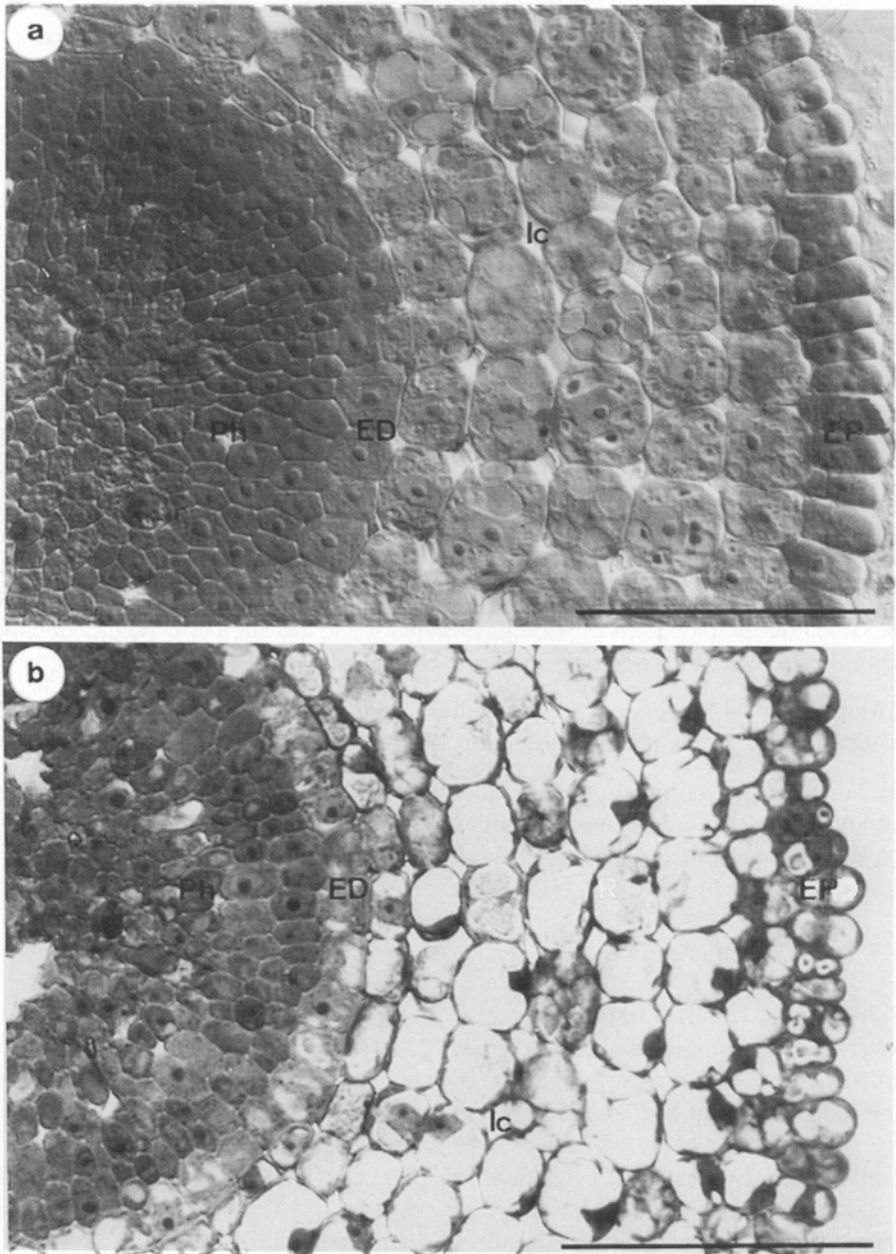
The decrease in electron density within the matrix of the mitochondria of salt treated plants seems to reflect a degeneration of the mitochondria (Öpik, 1965, 1966; Schwab *et al.*, 1969; Koyro, 1997). In root tips of salt-sensitive cultivars of *Agrostis stolonifera* and *Sorghum bicolor* x *S. sudanensis*, the sacculi of the mitochondria were swollen, whereas there was no saline effect on the ultrastructure of mitochondria in a salt-tolerant cultivar of *Atriplex stolonifera* and *Spartina townsendii* (Koyro, 1989; 1997; Smith *et al.*, 1982). Visible changes in the sacculi and / or endoplasmic reticulum also point to Ca-deficiency (Koyro and Stelzer, 1989) and may also be an indication of increased respiratory activity.

K-deficiency and NaCl salinity in salt-sensitive species can also lead to an increase in the number of mitochondria as well as to an increase in the number of microbodies (Hecht-Buchholz *et al.*, 1971; Hecht-Buchholz, 1983; Walker and Taiz, 1988; Koyro, 1997). The salt-induced increase in F-ATPase activity in *Sorghum* roots corresponds to the increase in the number of mitochondria in the cortex cells, and suggests an additional supply of energy for osmotic adaptation and for selective uptake and transport processes (Koyro *et al.*, 1993).

7.2.6 FURTHER CHANGES IN THE FINE STRUCTURE OF CELLS

In maize root tips, compared with the untreated control, treatment with $25 \text{ mol} \cdot \text{m}^{-3}$ NaCl for 8 h induced typical changes in the fine structure of the cells such as swelling of the proplastids, amyloplasts, and mitochondria (Hecht-Buchholz *et al.*, 1974; Munoz *et al.*, 1996; Khavari-Nejad and Mostofi, 1998). Huang and Van Steveninck (1990) have shown that salinity causes many plastids in cortical root cells of barley to adopt varying amoeboid shapes. In several subspecies of the sodiophil *Beta vulgaris* neither the K content nor the fine structure was changed (Koyro and Huchzermeyer, 1999). A close correlation between the rate of potassium efflux and the change in the fine structure was found thus reflecting the salt-susceptibility of the different plant species. It is suggested that there is a connection between the ability of plant species to retain potassium and their tolerance of high NaCl concentrations.

However, although several salt-induced changes were detected in rhizodermal cells of a highly salt-sensitive cultivar of *Sorghum bicolor* x *S. sudanensis*, the sole exception



*Figure 2: Light micrographs of cross sections of Sorghum roots 400 μm from the root tip. (a) Control (b) Plants cultured with 40 mol * m⁻³ NaCl. The scale bar represents 100 μm. Ph: Phloem, ED: Endodermis, Ic: Intercellular space, EP: Epidermis*

was the stroma of the plastids (Koyro, 1997). A change in the plastids seems not to be a precondition for low sensitivity.

7.3 Effect of NaCl on the exodermis, hypodermis and endodermis

Tracing the course of minerals through the plant from the rhizodermis to the leaf, it was shown that in *Suaeda maritima* and *Atriplex hastata* the root endodermis develops faster and the Casparian strip is significantly bigger after the addition of NaCl to the nutrient solution (Kramer *et al.*, 1978; Hajibagheri *et al.*, 1985).

It was possible to demonstrate the fast development of a Casparian strip in the exodermis, the hypodermis and the endodermis of many monocotyledoneous species (Peterson and Perumalla, 1984; Petersen, 1988; Perumalla *et al.*, 1990). The periblem originates in these layers. Salinity often leads to an additional deposition of suberin, lignin and callose into the periclinal and anticlinal cell walls (Clarkson *et al.*, 1987; Koyro *et al.*, 1993) and to a build-up of transfer cells (Koyro *et al.*, 1993). Moreover, Nishizawa and Mori (1984) have demonstrated the presence of vesicular (pinocytotic) transport from the plasmalemma to the tonoplast. These correlations point to functions of these three cell layers as physiological sheaths, and are often discussed in connection with the transport of water and the uptake of minerals.

The build-up of the endodermis with its Casparian strip forces solutes and solvent water to pass a membrane before entry to the central vein.

In cases of NaCl-salinity, a high selectivity of K over Na at the plasmalemma of these cell types can help a plant to avoid an excessive Na- or Cl- uptake and could therefore be partly responsible for tolerance to high NaCl-salinity.

7.4 Anatomical structure of the stele

Salinity induced structural changes in the stele (Gadallah and Ramadan, 1997). Few xylem vessels with smaller size were noticed in roots of stressed plants, while salinity enhanced xylem disstructure in leaves. Both effects seem to increase resistance to water transport to the leaf and help to diminish the overall water loss by the plant. The most interesting feature of the growing root tip, also called the absorption zone for water and most nutrients, is the xylem parenchyma (Kramer, 1983). These cells surround the proto- and metaxylem vessels, are relatively rich in cytoplasm and have prominent mitochondria and endoplasmic reticulum (Läuchli *et al.*, 1974).

The higher salt tolerance of certain cultivars of barley and citrus is related to a more effective restriction of shoot transport of both Na^+ and Cl^- (Greenway and Munns, 1980; Maas, 1993). Transport through the plasmalemma of the xylem-parenchyma cells into the apoplast of the xylem vessel enables the plant to control the composition of the xylem sap. Studies with X-ray microanalysis have shown that the concentrations of potassium in the xylem parenchyma and mature xylem vessels are significantly different (Läuchli *et al.*, 1971).

In cells of salt-stressed *Atriplex hastata* roots, the volume fraction of the cytoplasm to vacuole was higher than in control plants and increases in the number of mitochondria and cisternae of rough endoplasmic reticulum were observed (Kramer, 1983). These changes were explained by the assumption that transport processes such as the energy consuming exclusion of sodium are intensified and that the endoplasmic reticulum represents an intracytoplasmic compartment which can serve in the sequestration of ions within the cytoplasm.

For some species it is known that the xylem sap can be controlled secondarily during upward (or downward) transport into (from) the shoot. Some species show build-up of transfer cells with cell wall protuberances in cells of the xylem parenchyma (Kramer *et al.*, 1977; Kramer, 1983) or companion cells (Figure 3).

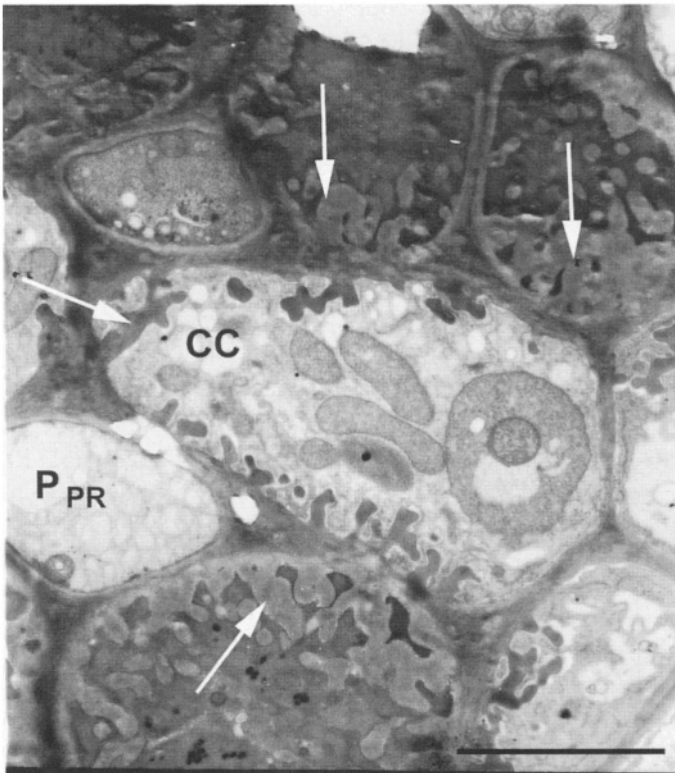


Figure 3: Electron micrographs showing companion cells (CC) and protophloem (P_{PR}) in salt-treated (500 mol * m⁻³ NaCl) *Aster maritima* leaves. The transfer cell shows the typical invaginations (cell wall protuberances, see arrows) of the inner cell wall. The scale bar represents 10 µm.

An overall assessment of the occurrence of transfer cells, their structure, development and role in the plant has been presented by Pate and Gunning (1972). The physiological activity of the transfer cells is clearly documented by the high number of mitochondria (Landsberg, 1982), which must be directly involved in the supply of energy to the (selective) ion transport process during H^+ expulsion. The transfer cells of the xylem parenchyma are discussed within the context of high substrate-salinity as locations of enhanced K^+/Na^+ -discrimination (Kramer, 1983). Sodium may be stored in surrounding cells or excreted into the soil.

Within the stele the most significant changes were observed in the xylem parenchyma cells. However, the salt tolerance of a plant additionally depends on its ability to regulate the movement of substances in the xylem as well as in the phloem. A high level of ATPase activity was induced by NaCl salinity in the tonoplast and plasmalemma of companion cells of *Sorghum* roots (Koyro, 1989) together with increases in the amount of vesicles. NaCl-salinity leads in several species to a rapid increase in the Na-concentrations in the phloem sap (Munns *et al.*, 1986, 1988). The high level in ATPase activity and small vesicles in the companion cells of *Sorghum* has been discussed by Koyro (1989) in connection with the recirculation of Na from the shoot to the root to minimize Na-accumulation in the shoot tissue of this salt-excluding species. This interpretation is underlined by the fact that salinity did not increase ATPase activity in the companion cells of the salt-tolerant species *Spartina townsendii*, a typical salt includer.

7.5 Influence of NaCl on leaf structures

Salinity has been shown to affect the time and rate of germination, the size of plants and leaf size, and overall plant anatomy. Succulence is one of the most common features of halophytes after exposure to salinity (Poljakoff-Mayber, 1975). In many plant species, it is especially the thickness of the spongy mesophyll and/or the palisade layer that increases with increasing salinity. It is often considered to be an adaptation, which reduces the internal salt concentration. However, many halophytes apparently reduce their internal salt content with the aid of salt glands or by salt exclusion on the part of roots, as in some mangroves (Chapter 3 and 6).

Waisel (1972) and Flowers *et al.* (1986) ascribe to salinity the following various effects on leaves, including the morphological and anatomical changes typical of halophytes:

Increase in succulence, leaf thickness and leaf area per plant, decrease in leaf number, changes in number and size of stomata, thickening of the cuticle, earlier occurrence of suberization and lignification, inhibition of differentiation, and changes in stelar diameter and number of xylem vessels.

There have been several reports on the structure of the cuticle as revealed by transmission electron microscopy, showing fine lamellation of the cuticle proper (Wattendorf and Holloway, 1980). Both cuticle and cell wall of the epidermal cells of leaves of *Suaeda maritima* showed a considerable increase in thickness in plants grown

in the presence of $340 \text{ mol} \cdot \text{m}^{-3}$ NaCl as compared with plants grown without added sodium chloride (Hajibagheri *et al.*, 1983).

There have been several investigations of the relationship between mesophyll cell size and leaf photosynthetic rates. Negative correlations between the two parameters have been reported for several species of both monocotyledonous and dicotyledonous plants (Dunstone and Evans, 1974). Stomatal resistance increases, but this is probably a consequence of decreased stomatal frequency rather than of a decrease in mean pore diameter.

Mesophyll resistance to the gas exchange increases under saline conditions as a consequence of structural changes in the mesophyll cells. It has been suggested that the main reason for this increase is a reduction in the volume fraction of intercellular spaces (Keiper *et al.*, 1998).

7.5.1 CHLOROPLASTS

The most obvious changes were observed in the chloroplasts (Hajibagheri *et al.*, 1985; Markovska *et al.*, 1995; Li and Ong, 1997; Utrillas and Alegre, 1997; Keiper *et al.*, 1998; Khavari-Nejad and Mostofi, 1998). The chloroplasts were aggregated, swelling occurred in the granal and fret compartments, and accumulation of lipid droplets could be identified. In plants grown at -1.1 to -1.9 MPa complete distortion of chloroplast structure occurred, there was no sign of grana and thylakoid structures in the chloroplasts, and other membranes were wrinkled. Swelling of the compartments of the grana and of the frets can progress to such an extent that the chloroplasts seem to be granaless. These changes were accompanied in some cases by a decrease in photosynthesis, and by cup shape and horseshoe shape of the chloroplasts (Li and Ong, 1997; H.-W. Koyro, unpubl. results). It was also possible to induce *in vitro* swelling of isolated chloroplasts at low osmotic potentials. (Blumenthal-Goldschmidt and Poljakoff-Mayber, 1966). The addition of ionic additives to the fixative also caused significant swelling of thylakoid membranes in chloroplasts (Hwang and Chen, 1997).

Halophytes such as *Atriplex hastata* or *Suaeda maritima* showed a dramatic accumulation of starch in their chloroplasts under saline conditions (Hajibagheri and Flowers, 1985; Hajibagheri *et al.* 1985). It is clear that the production of organic matter is stimulated under saline conditions and that this starch undoubtedly contributes to this effect. However, since the salinized plants are growing at a near maximal rate, it suggests that fixed carbon is not a limiting factor and that starch accumulation may be the consequence of reduced translocation.

Photosynthetic oxygen evolution in the chloroplasts of *Suaeda maritima* (Hajibagheri *et al.*, 1983) and chlorophyll synthesis in tobacco leaves were related to the ion content of the chloroplast (Motsan *et al.*, 1988). It was shown that an abnormally high internal Na^+ to K^+ or Na^+ to Ca^{2+} concentration and high concentrations of total salts inactivate enzymes, change plasma membrane permeability, and inhibit protein synthesis and photosynthesis (Drew, 1998).

High NaCl concentrations and maximum rates of oxygen evolution (coupled and uncoupled) were detected in the chloroplasts when plants were grown in $340 \text{ mol} \cdot \text{m}^{-3}$ NaCl. However, it seems unlikely that the observed stimulation of oxygen evolution by NaCl is of adaptive significance with respect to salinity tolerance, since similar NaCl optima have been recorded for a number of halophytic and non-halophytic species (Kinzel, 1982). For the halophyte *Avicennia marina*, it was shown that chloride apart from being an essential co-factor in oxygen evolution, was required at high concentrations for optimum photosystem II activity (Critchley, 1982).

In the chloroplasts of *Suaeda maritima*, *Atriplex hastata* and *Avicennia marina*, the large amounts of matrix and the numerous ribosomes were not affected by salinity. However, in spite of severe ultrastructural changes, no disturbances in the light reactions of these chloroplasts could be detected (Hajibagheri *et al.*, 1984).

7.5.2 MITOCHONDRIA

There are relatively few investigations of the effect of salinity on the fine structure of mitochondria in leaf cells (Siew and Klein, 1968). However, this respiratory organelle shows conspicuous structural damage after salt treatment with $100 \text{ mol} \cdot \text{m}^{-3}$ NaCl such as in *Agrostis stolonifera* (Smith *et al.*, 1982). Under these conditions the mitochondria became deficient in cristae, swollen and vacuolated. The mitochondria in leaf cells of *Atriplex* plants and in the cells of the salt glands of *Avicennia marina* and *Tamarix aphylla* grown under salinity appeared to be less electron-dense, with their cristae being swollen (Blumenthal-Goldschmidt and Poljakoff-Mayber, 1968). Observations of the mitochondria of leaf cells from salt (addition of $340 \text{ mol} \cdot \text{m}^{-3}$ NaCl) and non-salt plants of *Suaeda maritima* indicated variation in both size and shape (Hajibagheri *et al.*, 1985). A conspicuous feature of these experiments was the increase in the number of mitochondrial profiles per leaf cell section in salt as compared to non-salt plants. The number of mitochondrial profiles per mesophyll cell section increased from 3.4 in control plants to 7.2 when plants were grown under saline conditions. Ultrastructural changes in leaves of *Cynodon dactylon* under water stress also included an increase in the number of mitochondria per mesophyll cell (Utrillas and Alegre, 1997). The matrix of these mitochondria appeared less electron-dense. In pea leaves sodium chloride toxicity is associated with higher generation of superoxide radicals which suggests that an oxidative stress is involved at the level of mitochondria (Hernandez *et al.*, 1993).

Gametophytes of the mangrove fern *Acrostichum aureum* cultured in solutions containing 0.7% or 1% NaCl exhibited cup shape, horseshoe shape, ring shape, round or amoeboid mitochondria (Li and Ong, 1997). The mitochondria in these experiments seemed to be more resistant to salt stress compared with chloroplasts and there was no direct relationship between changes in respiration rate and changes in mitochondrial shape. The changes were reversible in all cases.

7.5.3 NUCLEI, ENDOPLASMIC RETICULUM, GOLGI APPARATUS, PLASMALEMMA AND TONOPLAST

The nuclei, the endoplasmic reticulum, and the golgi apparatus in leaf cells of plants grown under salinity seemed to be unchanged. There were only very few examples of the double membrane of the nuclei being swollen (Blumenthal-Goldschmidt and Poljakoff-Mayber, 1968; Strogonow, 1970). However, the tonoplast in leaf cells of the halophyte *Atriplex* spec. and the glycophytic species tomato, barley and cotton showed many inward coils and folds and the plasmalemma appeared wavy and retracted from the cell wall. This feature of the plasmalemma became more pronounced with increasing salinity.

7.5.4 DEGREE OF VACUOLATION OF LEAF CELLS

Extensive vacuolation appeared in leaf cells. The vacuole increased in size relative to the cytoplasm and an extensive network of transvacuolar strands developed in cells of *Nicotiana tabacum* as a response to NaCl - supply (Chang *et al.*, 1996). These changes altered the surface contact between the cytoplasm and the vacuole substantially. Many changes in membrane proteins could also be identified after NaCl - adaptation of tobacco.

An increase in succulence has long been held to be an important response of plants to salinity (Jennings, 1976; Munns *et al.*, 1983; Hajibagheri *et al.*, 1984). The vacuoles are the sites for the major component of osmotic adjustment within halophyte leaf cells and NaCl is mainly stored in the vacuole. An increase in the volume fraction of the vacuole points to an increased storage capacity for NaCl.

7.5.5 MICROBODIES

The microbody volume per cell section increased under saline conditions in leaf cells of the halophyte *Suaeda maritima*, but the number per cell was insensitive to salt (Hajibagheri *et al.*, 1985). The leaf microbodies were characteristically appressed to chloroplasts and mitochondria. The equivalence of the microbodies to peroxisomes was shown cytochemically by employing diaminobenzidine for the localisation of catalase. There was no clear difference in the electron-dense reaction product, and no crystalline inclusions were seen in microbodies of *Suaeda* plants, either under salt or under non-salt conditions. This is consistent with results found for leaves of non-halophytic plants (Frederick and Newcomb, 1969). However, the ratio of the number of mitochondria to microbodies in leaves of *Suaeda maritima* showed an increase in growth of plants under saline conditions, which is interpreted by the authors as a possible change in the balance of mitochondrial respiration to photorespiration (Hajibagheri *et al.*, 1985).

7.6 Conclusions

Several examples of structural adjustment of halophytic and glycophytic plant species are presented in this chapter. It is concluded that the salt tolerance of a plant depends on a combination of structural adjustments to NaCl-salinity in which particular reaction patterns enable the plant to cope with the conditions prevailing in the edaphic environment. There are only very few examples of qualitative differences between glycophytic and halophytic species, such as bladder hairs in *Atriplex hastata* or salt glands in *Spartina alterniflora*, *Laguncularia racemosa* or *Avicennia marina* (Kramer *et al.*, 1978; Koyro, 1989, 1997; Chapter 3).

It was necessary to restrict the review of ultrastructural effects of salinity to selected organs (roots and leaves) and cell types of higher plants. However, it makes sense to extend examination by analogy to other plant organs such as the stem, the leaf petiole, the flower or the fruit or even to evaluate structural information on the division of labor between juvenile and adult leaves (Marschner, 1995).

There is a high demand for further investigation of ultrastructural effects caused by NaCl salinity. There are, for example, only very few articles available on the effects of NaCl salinity on the division of labor in a leaf areole containing sieve elements and exhibiting differing degrees of development. The influence of NaCl on carbohydrate metabolism, shows clearly that there is a high demand for regulation and protection, particularly in the area of phloem loading and unloading (Van Bel, 1989; Van Bel and Gamalei, 1991).

Additionally, the transition between different plant organs often shows a markedly difficult structural build-up. Obviously, regulative or protective structures seem to be especially important at these locations. Much more attention needs to be paid in the future to the regulation of NaCl-salinity at critical locations such as (a) the root base, (b) the connection between stem and leaf petiole and (c) the connection between leaf petiole and lamina. In trees or woody species, the function of the periderm (lenticel) in the regulation of the uptake of NaCl-saline aerosol needs to be integrated into the overall picture of regulation in order to avoid excessive internal NaCl-concentrations.

Immunological techniques, single-cell analysis of water and solute relations, the molecular basis of mechanisms enabling a plant to exhibit high or low salt tolerance, and ultrastructural investigations – all these are employed in many cases solely to describe the reactions of a plant to substrate-salinity. The results can be misleading because they are based on a lot of hypothetical assumptions (Munns, 1993).

If we want to understand the fine regulation and different strategies adopted by individual plant species to avoid salt injury special attention has to be paid to the overall picture of cytological (e.g. ultrastructural) changes, the integration of a cell into the surrounding tissue, the integration of a tissue into a plant organ, and last but not least, the division of labor between different organs.

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7.7 References

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CHAPTER 8

INTRA- AND INTER-CELLULAR COMPARTMENTATION OF IONS

A Study in Specificity and Plasticity

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Abstract

Accumulation of sodium salts by higher plant tissues contributes to osmotic adjustment to increased external salinity. This crude statement masks subtle and varied differences in behaviour between the main intracellular compartments (cell wall, cytoplasm and vacuole), and between cell types. The cytosol, is characterized by a marked selectivity for K^+ and HPO_4^- (in P deficient plants), but against Ca^{++} , Na^+ , Cl^- , NO_3^- and H^+ and organic acids. These solutes are largely occluded in the vacuole. In hyperosmotic conditions compatible organic solutes accumulate in the cytoplasm to achieve osmotic equilibrium across the tonoplast. Turgor homeostasis is an important component of the plant's response to salt stress, but this is dependent on the regulation of changes in both the intracellular and cell wall solutes. There are also marked differences in the ions accumulated in vacuoles of leaf epidermal, mesophyll and other cell types that appear to be cell- and species-specific.

8.1 General introduction

The salinity of the marine environment is dominated by high concentrations of Na^+ and Cl^- , although K^+ , Ca^{++} , Mg^{++} and SO_4^{--} are also significant contributors to the total solute concentration (Table 1). In terms of water and osmotic potentials, these chemical concentrations equate to *circa* - 2.4 MPa (-24 bars) and 1000 mosmol kg^{-1} . In terrestrial saline habitats the individual ionic concentrations, water potential and pH of the substrate are much more variable. Further the osmotic potentials arising from salt accumulation may be superimposed on low soil matrix potentials due to arid or semi-arid conditions. In both the marine and the terrestrial habitats, the high transpirational

demand may also create further strong thermodynamic gradients of water potential between the plant and soil and the atmosphere. In studies on halophytes from salt marshes (Albert and Kinzel, 1973; Gorham *et al.*, 1980) it was observed that the leaves usually contained high concentrations of Cl^- and Na^+ (Table 2). It was appreciated that such large accumulations of Na^+ and Cl^- were required to counteract the low external water potential and maintain an adequate gradient of water potential (Ψ), both to help ensure tissue water retention and to generate positive cell turgor.

TABLE 1. Approximate composition of seawater.

Parameter	Value	Units
Osmolality	1000	mOsmol kg ⁻¹
Osmotic Pressure	2.5	MPa
Electrical Conductivity	55	dS m ⁻¹
Chlorine	542	mmol kg ⁻¹
Sodium	480	mmol kg ⁻¹
Magnesium	53	mmol kg ⁻¹
Sulphur	27	mmol kg ⁻¹
Calcium	10	mmol kg ⁻¹
Potassium	10	mmol kg ⁻¹
Bromine	1	mmol kg ⁻¹

In this chapter we will be exploring the increasingly detailed dissection of these basic observations that has been achieved in recent years both in halophytes and in glycophytes subjected mainly to NaCl salinity. The basic observation that high concentrations of salt are accumulated in the mature leaves of many halophytes and glycophytes is unchallenged, but it has become clear that this masks the marvelously intricate sub-cellular, cellular, organ and often species-specific responses of plants to salinity. Plants contrive to combine some highly conserved solute requirements at the cytoplasmic level with great plasticity at vacuolar and tissue levels.

In order to evolve from the gross picture of NaCl accumulation revealed in Table 2 to our current understanding, plant scientists have had to confront and overcome many technical problems. This chapter will therefore allude to, but not elaborate upon, these technical advances, most of which have occurred since the mid 1970's.

The first part of this chapter will consider our broad understanding of intracellular and intercellular ion compartmentation with special reference to K^+ and Na^+ and the principles that underpin it. The second part of this chapter will describe, briefly, two examples, [i] ion distribution in relation to root growth and [ii] ion distribution as related to leaf structure and function in greater detail.

TABLE 2. Ion contents (mol m⁻³ plant water) of plants from saline habitats. Data from an inland saline lake¹ (Albert and Kinzel, 1973), an estuarine salt marsh² (Gorham *et al.*, 1980) and a mangrove swamp³ (Popp, 1984).

Species	Na	K	Ca	Mg	Cl
<i>Phragmites communis</i> ¹	11	235	64	66	161
<i>Juncus maritimus</i> ²	100	234	17	33	142
<i>Scirpus maritimus</i> ²	144	115	20	19	183
<i>Puccinellia maritima</i> ²	160	159	39	69	229
<i>Glaux maritima</i> ²	237	103	25	62	225
<i>Spartina anglica</i> ²	346	159	56	38	315
<i>Ceriops tagal</i> ³	386	159	45	255	550
<i>Avicennia marina</i> ³	520	160	-	125	556
<i>Suaeda maritima</i> ²	547	74	38	131	532
<i>Rhizophora lamarckii</i> ³	558	86	34	135	525
<i>Spergularia media</i> ²	615	70	28	85	481
<i>Salicornia europaea</i> ²	820	50	19	115	965
<i>Salicornia europaea</i> ¹	863	159	1	37	578

8.2 Intracellular compartmentation model

The intracellular compartmentation of ions in higher plants was first postulated in the early 1970's by Greenway and Osmond (1972) and Flowers (1972), although the hypothesis had antecedents in the literature on marine invertebrates and fungi. The concept arose from studies showing that enzymes extracted from the leaves of salt-accumulating halophytes were inhibited by NaCl concentrations (and indeed those of other uni-univalent salts) significantly below the levels found in healthy leaves (Flowers *et al.*, 1975,1976,1978).

Hard on the heels of these observations came the first papers reporting the accumulation of organic solutes such as proline (Stewart and Lee, 1974) and betaine (Storey and Wyn Jones, 1975, 1977; Storey *et al.*, 1977) in higher plants in response to graded salinity. Evidence that betaine occurred in high concentrations in the leaves of halophytes had been published in the literature of the Bohemian sugar industry (Stanek and Domin, 1909), while salt-induced accumulation of proline and betaine had been observed in marine invertebrates in the mid 1950s (see Schoffeniels and Gilles, 1972).

Flowers *et al.* (1977) in an influential review collated the early evidence that NaCl is preferentially accumulated in the metabolically, relatively inert vacuole while non-toxic organic solutes, now usually referred to as compatible cytosolutes, were selectively accumulated in the cytoplasm. However this review did not address the issues surrounding the distribution and function of K⁺ under saline conditions.

Halophytes often have high leaf Na/K ratios (Table 2) and the total K^+ level decreases with increasing external NaCl. It has been widely observed (e.g. Storey and Wyn Jones 1978 a, b and 1979) that in many species increasing external $Na^+(Cl^-)$ will result in a sharp decline in leaf K concentrations and it has been suggested that Na could induce K-deficiency in some species, an observation confirmed by more recent work (Bernstein *et al.*, 1995; Botella *et al.*, 1997). However there were strong indications of a more complex relationship. Marschner (1971) and others had shown that Na should 'spare' the K requirement of some plants including a number of halophytes, e.g. sugar beet. Marschner (1995) and Flowers and Läuchli (1983) contain discussions on the substitution of potassium by sodium in different species. In no case, however, was there evidence that Na is required as an essential macronutrient in higher plants or indeed in lower plants, but it has been shown to be a beneficial nutrient in some halophytic C_4 species (Brownell, 1979; see also Chapter 16). This is in marked contrast to animals where Na is an important macronutrient.

Halophyte physiology was, however, faced with a major paradox (see also Chapter 3). Not only could Na spare the requirement for K but vegetative growth, especially on a wet weight basis, of a number of halophytes was promoted by levels of sodium salts up to about 150 to 200 mol m^{-3} . This behaviour is usually associated with an increase in leaf succulence and is characteristic of dicotyledonous species. Many broad comparisons between divergent species going back to the 1930 and 40s (e.g. Collander, 1941) showed that the more tolerant halophytic species had a greater capacity to carry high leaf salt loads and had higher leaf Na/K ratios. Work from Lessani and Marschner (1978) also suggested that leaf Na was less mobile in these species. Nonetheless, in many comparative studies on cultivars of the same species it was nearly always observed that the cultivar better able to exclude Na and Cl from its leaves was the more tolerant (e.g. Greenway, 1962; Storey and Wyn Jones 1978 a). Much recent work has confirmed this in comparisons of related species (see Läuchli, 1999 for references) and in more stringent work on physiological genetics of selfed lines selected from a given cultivar (Yeo *et al.*, 1988; Salam *et al.*, 1999). So it was necessary to explain why many halophytes could apparently accommodate high leaf NaCl concentrations and high Na/K ratios although salt (Na and Cl) exclusion and the maintenance of lower K/Na ratios were traits associated strongly with enhanced tolerance in most glycophytes.

The application of Pitman's (1965) selectivity ratio [$S_{K/Na}$] also indicated a more subtle relationship between sodium and potassium uptake. In tolerant species, although the crude leaf K/Na ratio declined, K^+ selectivity actually increased with external salinity especially in tolerant plants (see Storey and Wyn Jones, 1978 a, b), indicating that plants contained specific mechanisms to maintain K levels in the presence of high external Na concentrations. Paradoxically the growth of many halophytes is more damaged by equivalent and indeed rather lower levels of K salinity than by sodium salts (Mozafar *et al.*, 1970).

An examination of the comparative literature from a range of terrestrial and marine eukaryotic organisms, and some limited data on plants, indicated a strong cytoplasmic K^+ -selectivity and a relatively constant K activity with Na salts occluded in the bathing

fluids, e.g., serum or blood in animals, or in vacuoles in plants (Wyn Jones and Pollard, 1983). In order to try and provide a coherent explanation for these apparently contradictory observations, the Bangor group suggested that K and Na have distinct roles and made a simple postulate, in line with Marschner (1971), that K was selectively and specifically accumulated in the cytoplasm while Na and Cl were occluded in the vacuole. On this basis Wyn Jones *et al.* (1977) proposed a generalized, semi-quantitative model for solute distribution and cytoplasmic ion homeostasis that also fitted with the putative ion selectivities at the plasma and vacuolar membranes (Figure 1). Recent detailed work on the selective ion transport mechanisms at the plasma membrane and the tonoplast has enormously expanded our understanding and remains consistent with the early model. This will not be discussed further in this chapter, but see Chapters 18 and 19. It was also suggested in 1977 that the model could be extended to phloem-fed tissues and indeed was true of most eukaryotic cells. Yancey *et al.* (1982) expanded and broadened the concept to incorporate urea-accumulating fish and more recent work has confirmed that very similar mechanisms are found in mammalian kidneys (Bagnasco *et al.*, 1986, 1987) and indeed eubacteria (Csonka, 1989). Thus the breadth and applicability of the hypothesis have been greatly strengthened. The exceptions to the general model are the Archaeobacteria that are found in the most extreme environments on this planet and have specialized cytoplasmic enzymes and membranes stable in solutions of very high ionic strength.

The main postulates of the original model were and remain:

- Preferential cytoplasmic accumulation of K^+ to concentrations in the range 100 to 150 mol m^{-3} with Na^+ and Cl^- limited to 30 to 50 mol m^{-3} or lower.
- Excess Cl^- and Na^+ accumulated in the vacuoles to achieve gross osmotic adjustment thus, as noted earlier, dominating most tissue analyses (Table 2, Figure 2).
- Osmotic equilibrium across the tonoplast under hyperosmotic conditions is achieved by the accumulation of compatible cytosolutes, e.g. betaine, proline, sorbitol, quebrachitol, (usually characteristic of a particular taxonomic group) at cell osmotic potentials above about 350 to 400 mosmol kg^{-1}

One deficiency of the model is that it considers only the cytosol, whereas a large part of the volume of the cytoplasm consists of organelles such as the nucleus, chloroplasts and mitochondria. In the case of chloroplasts there is evidence for high K/Na ratios and accumulation of the compatible solute glycinebetaine (Schroppel-Meier and Kaiser, 1988; Robinson and Jones, 1986).

The underlying reason for the K^+ -selectivity of the cytosol was not immediately apparent (Wyn Jones *et al.*, 1977, 1979) although it had been suggested in the early 60's that, in mammalian cells, their K-selectivity could be related to the K requirements of protein synthesis (Lubin and Ennis, 1964). Gibson *et al.* (1984) showed that the cytoplasmic K requirement could be related, both quantitatively and qualitatively, to the K-specificity of mRNA translation in all eukaryotic cells and to the

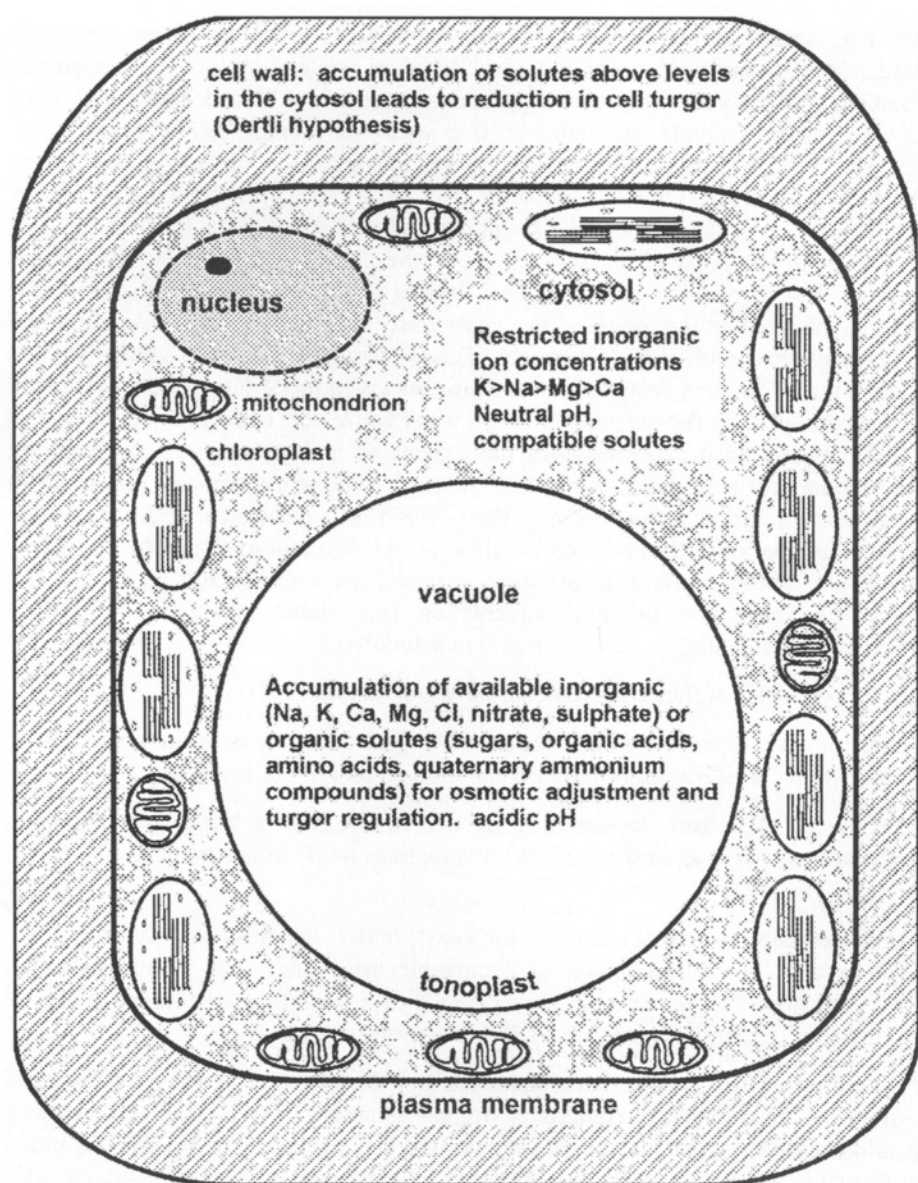


Figure 1. Model of intracellular solute compartmentation. The model predicts that the cytoplasm should have K^+ concentrations in the range 100 to 150 mol m^{-3} with Na^+ and Cl^- limited to 30 to 50 mol m^{-3} or lower. Metabolic considerations suggest low concentrations of calcium, magnesium, phosphate, sulphate, nitrate and malate in the cytoplasm. Excess ions and other solutes (including potassium) accumulate in the vacuole and possibly in the cell wall. Osmotic equilibrium across the tonoplast is achieved by the accumulation of compatible cytosolutes at cell osmotic potentials above about 350 to 400 mosmol kg^{-1} .

stability of the ribosomal complex. It is important to emphasize that this mechanism for translation is highly conserved in all eukaryotic cells with animal messages capable of being translated by the plant ribosomal complex and *vice versa*.

In animal systems both low K and changes in K/Na ratios have been shown *in vivo* and *in vitro* to affect the integrity of the translation process (Wool, 1979). While protein synthesis has frequently been shown to be inhibited by K-deficiency in plants and by salinity (Flowers and Dalmond, 1992; Koch and Mengel, 1977), quantitative data linking cytoplasmic K activities in plant cells to the rate of *in vivo* protein synthesis at the cell level are missing in higher plants.

Much work has been directed at proving or falsifying the hypothesis of solute compartmentation and cytoplasmic homeostasis by direct analysis, especially in angiosperms and the extreme halophytic green alga, *Dunaliella salina* (viz., Bental *et al.*, 1988) where serious doubt had been cast on the validity of the model. Methods have had to be developed to determine quantitatively and specifically solute concentrations [activities] in the cytosol and vacuole of various cell types and this has proved a significant challenge. Figure 2 summarizes the ranges of ion concentrations reported in plant cytoplasms and vacuoles for a range of external salinities.

However the model also had more easily testable inferences:

- that individual cytosolic enzymes and organelles, *e.g.* mitochondria and chloroplasts, should be metabolically active in high concentrations of the putative compatible solutes;
- that these compatible solutes should be accumulated in halophytes under conditions where growth is stimulated by modest saltness, *i.e.*, accumulation is clearly part of an adaptive rather than a toxic response;
- that enzymes located in the vacuole, dominantly hydrolases, should be relatively salt insensitive at least in halophytic species.

These inferences have all been tested and confirmed (Flowers *et al.*, 1986; Gorham *et al.*, 1985). Indeed studies on the effects of compatible solutes showed that in certain circumstances, but not universally, these solutes could protect cytoplasmic functions against inhibition by salts (both NaCl and KCl) and other perturbations such as high temperature and freezing. This has lead to some confusion in the literature and with suggestions that the cytoprotective activity was their major (only) role ignoring the physical need for a sufficient cytosolic solute potential (Nash *et al.*, 1982; Paleg *et al.*, 1984; Winzer *et al.*, 1992; Zhao *et al.*, 1992) to ensure osmotic equilibrium across the tonoplast. The function and metabolism of compatible solutes are discussed in Chapter 9.

To return to the whole plant and tissue observations on K and Na relations alluded to earlier, the model implies that the greater capacity of halophytes, especially halophytic chenopods to use/respond to NaCl and the greater capacity of Na to spare K in such species is related to their capacity to use Na as a 'cheap' vacuolar osmoticum while maintaining a healthy, active cytoplasm by selective K and compatible solute

accumulation in this compartment. Similarly it might be postulated that the greater toxicity of K salts to such plants may be related to the orientation and selectivity of the tonoplast carriers so that a steep tonoplast K/Na gradient [K-cytoplasmic/Na-vacuolar] can be maintained.

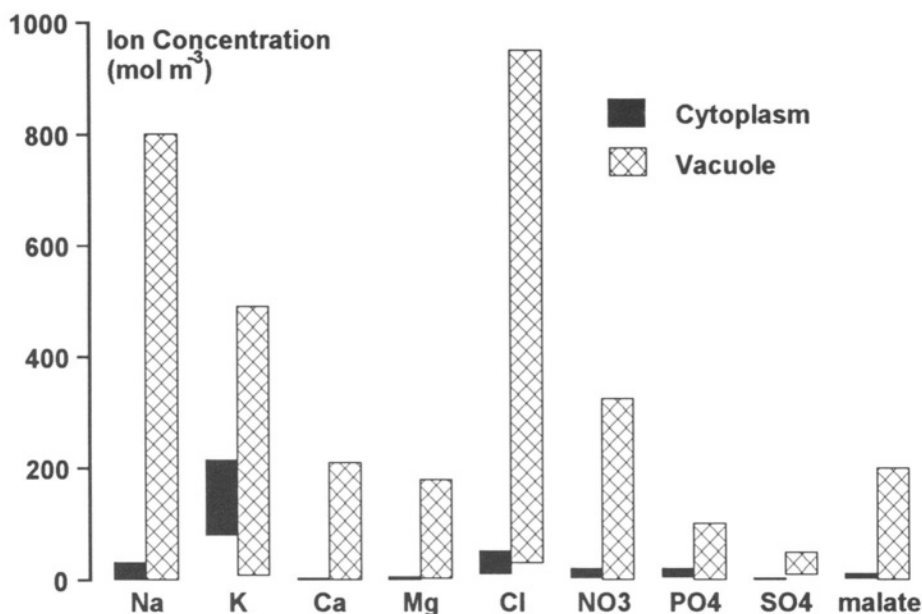


Figure 2. The ranges of ion concentrations in cytoplasm (solid bars) and vacuoles (hatched bars) for a variety of plants grown under a variety of salinities. Data from Bell *et al.* (1995), Devienne *et al.* (1994), Fricke *et al.* (1996), Leigh (1997), Leigh and Wyn Jones (1986), Preisser *et al.* (1992), Wyn Jones (1999) and Zhen *et al.* (1991). To quote from Leigh (1997) 'It now seems clear that there is no lower limit to the concentration to which a solute concentration may decrease in the vacuole [under conditions of deficiency], i.e. no threshold lower value (Leigh and Wyn Jones, 1984), and there is also evidence to suggest that there is no upper limit providing there is regulation of turgor'. This Figure does not show the very high values reported for cytosolic Na⁺ and Cl⁻ in some members of the Chenopodiaceae (see below, Table 3).

8.3 Cytosolic homeostasis and the variability of cell contents

While the original postulate (Wyn Jones *et al.*, 1977) was directed at the distribution of K, Na and Cl and organic solutes in higher plant cells, it was apparent that distribution of other ions and solutes might be illuminated by similar considerations. Williams (1970) noted that in animals Na, Cl, Ca and SO₄ are predominately extracellular or more accurately 'acytoplasmic'. Extrapolating these concepts to plants and regarding the vacuole as having some of the characteristics of extracellular fluid, we can recognize ion pairs that are respectively cytoplasmic or vacuolar, e.g. K⁺/Na⁺; Mg⁺⁺/Ca⁺⁺; inorganic phosphate (Pi) /Cl⁻, while NO₃⁻, organic acids, H⁺ are all characteristically vacuolar solutes. This hypothesis imposed specific constraints, as will

be discussed later, on the mechanisms by which higher plants and algae could adapt to salinity. Given the compelling evidence for a symplasmic continuum in many cell assemblages in higher plants (Läuchli, 1976) and that the phloem is essentially modified cytoplasm, although adapted to operate at a pH of about 8-8.2 (Raven, 1977; Gerendas and Schurr, 1999), it is logical to anticipate that solute selectivity of cytoplasm will have broad implications for nutrient ion function and distribution in all conditions, but especially in salinity.

Two broad approaches have been taken to establishing the ion activities/concentrations of the cytoplasm. The first has been to develop methods of sufficiently high resolution to analyze the very small cytoplasmic volume separately from the vacuole and the cell wall. Secondly tissues of naturally low vacuolation have been analyzed and the solute concentrations in cytoplasm of mature vacuolated cell inferred from them.

In the first category we can place efflux analysis, several variants of X-ray microanalysis and ion specific microelectrodes. The first was very valuable before more direct methods became available but was dependent on extrapolation from a mathematical model (e.g. Stelter and Jeschke, 1983) based on the assumption of homogeneity of the tissue. Problems with sample preparation and uncertainty about the precise volume activated by the beam have limited the value of X-ray microanalysis in relation to cytoplasmic ion concentrations. In some examples these problems were minimized by analyzing cytoplasmically enriched cells (Storey *et al.* 1983 a, b). In other work the use of freeze substitution or an inability to separate the cell wall and the cytoplasm may also have produced debatable analytical results (e.g. Harvey *et al.* 1981).

Early work with ion selective electrodes was also plagued by uncertainty about the location of the electrode tip in mature cells. Recently a triple barrelled electrode capable of simultaneously measuring pH, K^+ activity, and the membrane potential has been developed (Walker *et al.*, 1996), allowing identification of vacuolar and cytosolic samples and giving unambiguous values for cytosolic K^+ activity. It has provided clear evidence for K^+ homeostasis in the anticipated concentration range, when the activity results are corrected for the activity coefficient (about 0.75) of K^+ in the cytosolic milieu (Figure 3). In addition there is evidence of a strong degree of cytoplasmic H^+ and Pi homeostasis and of preferential NO_3^- accumulation in the vacuole (Leigh and Wyn Jones, 1986; Leigh, 1997) to confirm the broad applicability of the concept. The recent work of Serrano and his colleagues have provided molecular genetic evidence for the sensitivity of cytoplasmic enzymes to low concentrations of Na and strong indications of how cytosolic homeostasis is accomplished in micro-organisms (Serrano *et al.*, 1999). A large body of work, mainly on cells in culture, has provided further compelling evidence for both ion homeostasis and the selectivities proposed earlier (Niu *et al.*, 1995).

The indirect method of extrapolation from cytoplasm-rich, often juvenile tissues (e.g., apical meristems: Gorham and Wyn Jones, 1983), young carrot explants and embryos, pollen and floral structures: Gorham *et al.*, 1980, or even direct analysis of phloem sap) all give semi-quantitative support to the hypothesis in halophytes as well as glycophytes. There is, however, an element of circularity in this evidence. As

cytoplasmic-rich tissues (*e.g.*, apical meristems and developing embryos) are usually phloem-fed, and phloem sap is high in K^+ , Mg^{++} and Pi and low in Na^+ , Cl^- and free Ca^{++} but rich in sugars, sugar alcohols and compatible solutes, such results could be regarded self-fulfilling. But these relationships could be more profitably viewed as the close integration of the metabolic constraints implicit in the hypothesis of cytoplasmic solute selectivity and ion homeostasis with cell anatomy and tissue morphology (Wyn Jones 1999). Should the observations that seeds of, *e.g.*, barley (Greenway 1962), *Salicornia europaea* and other halophytes (O'Leary, 1984; Gorham *et al.*, 1986) and fruits (*e.g.* tomato: Rush and Epstein, 1980, 1981) are low in $NaCl$, be thought of as evidence for cytoplasmic selectivity or as a remarkable example of the way in which structure and physiology are integrated in plants, especially halophytes? Presumably it is both.

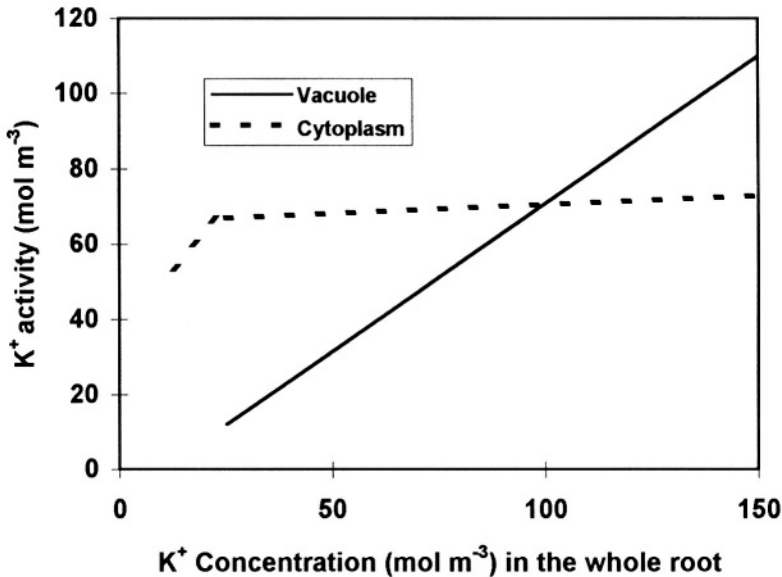


Figure 3. Changes in vacuolar K^+ activity and stability of K^+ activity in the cytoplasm of cortical cells of barley roots. The plants were grown at a range of K^+ concentrations in the nutrient solution, resulting in a range of whole root sap K^+ concentrations. Vacuolar and cytoplasmic K^+ activities were measured with triple-barrelled K^+ -selective microelectrodes. Data from Walker *et al.* (1996).

There is some direct evidence to show that cytoplasmic homeostasis is broken down by salinity or that the K/Na ratios are altered and that such changes can be related to differential tolerance. Comparisons of two maize cultivars (Table 3; Hajibagheri *et al.*, 1989) indicate that changes in the cytoplasmic K/Na ratios are directly related to tolerance. Data comparing two well-known halophytic grasses, *Puccinellia peisonis* and *Spartina townsendii*, with the relatively salt-sensitive crop species, *Sorghum bicolor* x *S. sudanensis*, also suggests that the capacity to maintain for cytoplasmic

high K/Na discrimination under saline conditions is greater in the halophytes (Koyro and Stelzer, 1988).

The original hypothesis of cytoplasmic/symplasmic homeostasis did not address the possibility of a dynamic change in the homeostatic set points and/or in K/Na selectivity ratios as the metabolic imperatives change, *e.g.*, in mature and senescing leaves or even in different tissues. Marschner (1995) summarized the evidence that in older tissues of natrophilic species K^+ can be replaced by Na^+ even in cytosolic and chloroplast functions. They contrasted this with a rigorous K requirement for cell division and differentiation. There is insufficient evidence to prove this contention. It was, however, recently pointed out that both the mean K tissue content and the mean turgor pressures are lower in root cells than in leaf cells (Wyn Jones, 1999). Thus the "homeostatic set points" may be tissue dependent. A similar suggestion has emerged from comparison of *Beta vulgaris* races selected over many generations to accumulate different quantities of sugar in the storage vacuoles (Tomos *et al.*, 1992). Other results suggest that leaf mesophyll cell cytoplasm has lower K/Na ratios than more juvenile cells and bundle sheath cells (Storey *et al.*, 1983 a, b). Alternatively salt load may overwhelm the control mechanisms in an unregulated way as the cell ages.

TABLE 3. Ion contents ($\mu\text{mol g}^{-1}$ dry weight), cytoplasmic ion concentrations (mol m^{-3}) and Na/K ratios calculated from efflux analysis for roots of two maize varieties grown in 50 mol m^{-3} NaCl. Data from Hajibagheri *et al.* (1989). Note that cytoplasmic Na^+ and Cl^- concentrations are outside the ranges shown in Figure 2.

Ion		LG11 (salt-sensitive)	Protador (salt-resistant)
Na^+	whole root ($\mu\text{mol g}^{-1}$ dw)	2153 \pm 234	1430 \pm 296
	cytoplasm (mol m^{-3})	142 \pm 10	79 \pm 3
K^+	whole root ($\mu\text{mol g}^{-1}$ dw)	550 \pm 33	759 \pm 45
	cytoplasm (mol m^{-3})	70 \pm 10	120 \pm 26
Cl^-	whole root ($\mu\text{mol g}^{-1}$ dw)	2000 \pm 198	1560 \pm 134
	cytoplasm (mol m^{-3})	563 \pm 34	360 \pm 23
Na/K ratio			
whole root		3.91	1.88
cytoplasm		2.03	0.66

8.4 Some cytological interactions

While many halophytes have very high vacuolar Na/K ratios and consequently tissue analyses, K^+ may be the dominant cation in some species. This is commonly observed in some marine macro algae (Kirst and Bisson, 1979) and in some halophytes, especially some members of the Poaceae, *e.g.*, *Puccinellia* sp. and *Leptochloa fusca*. It may be relevant that in these monocotyledonous species succulence does not increase substantially in response to salinity nor is growth stimulated by salt. These macro nutritional and cytological differences are incorporated into the concept of 'physio-

types' *vide* Albert and Kinzel (1973). However this is also primarily a distinction between monocotyledonous and dicotyledonous responses to salinity; there is undoubtedly a close relationship between NaCl growth promotion and succulence. Interestingly in *Puccinellia* the high leaf K level may be associated with the evolution of a second casparian strip in the root that must reinforce the barrier to apoplastic flow across the root (Stelzer and Läuchli, 1977 a, b).

Attempts to study the cytoplasmic compartmentation of ions, often focusing on the K/Na ratio and using x-ray microprobe analysis, revealed two somewhat unexpected observations. It became clear that solute concentrations in the cell wall could be high, validating, in the case of halophytes, an important prediction made by Oertli (1968). The use of the pressure probe in species such as *Suaeda maritima* has provided quantitative evidence for this phenomenon (Clipson *et al.*, 1985). Salt sensitivity in rice has also been attributed, in part, to the accumulation of ions in the cell walls (Flowers *et al.*, 1991). In salt-treated maize and cotton, however, there is evidence for low apoplastic Na^+ concentrations (Mühling and Läuchli, 2001 a, b), suggesting considerable variation between species (and ions?) for apoplastic salt accumulation. New techniques such as the use of ion-sensitive fluorescent dyes (Mühling and Sattelmacher, 1997; Mühling and Läuchli, 2001 b) and applications of the micro pressure probe (Tomos and Leigh, 1999) are contributing to a growing understanding of apoplastic (cell wall) solutes. Thus turgor pressure, which depends on the difference between the cell wall water potential and the cell osmotic potential, may be very low even when the tissue solute concentrations are high. It follows that gross tissue K^+ and Na^+ concentrations are poor indicators of the effects of salt on water relations, and that the control of ion fluxes into the cell wall is as significant as the better studied fluxes into other intra-cellular compartments. In this context it should also be noted that the responses of *Atriplex spongiosa* to NaCl and the uptake of the ions are dramatically affected by the atmospheric relative humidity, and therefore the transpirational gradient (Salim, 1989).

It was also apparent that individual ion concentrations in vacuoles are very variable (*e.g.*, Leigh and Storey, 1993; Fricke *et al.*, 1996; Prichard *et al.*, 1996). This is consistent with earlier observations that there are at least two distinct types of vacuoles, one with high salt contents and another with high organic solute concentrations (Van Steveninck *et al.* 1976). Thus it is totally misleading to treat vacuoles as inert balloons. In the case of some halophytes it was observed many years ago that the vacuoles of various specialized cells could act as NaCl sinks. An excellent example would be the bladder cells of *Atriplex* species (Freitas and Breckle, 1992; Storey, *et al.*, 1983 a; Chapters 3 and 16), but also specific salt (NaCl) sequestering cells with the characteristic plasma membrane invaginations of transfer cells are found quite widely (Koyro, 1997; Kramer, 1983; Kramer *et al.*, 1977; Chapter 7). In physiological terms all these mechanisms are part of the web of alternatives available to different species to retain high K/Na ratios in crucial tissues such as growing apices.

In the case of salt glands (see Chapter 3) cytological examination has revealed another important factor. The cell walls surrounding the salt accumulating cells are impregnated by a water-impermeable suberin, that effectively cuts off apoplastic water

and ion fluxes through those cell walls (Thomson *et al.*, 1988; Storey and Thomson, 1994). Chemically this structural barrier to apoplastic flow is similar to that in the casparian strip and in the rhizodermis of mature roots and to suberin in the leaf cuticle. Similar structures are found at the node between the base of the stem and the root, and between the developing seed and its supplying structures. As these suberized walls may still be traversed by cytoplasmic strands, plasmodesmata, the net effect is to ensure that symplasmic nutrient supply dominates. The orientation and density of these plasmodesmatal links are probably very important in determining the direction of solute flows. It is worth noting that even at modest salinities all higher plants are net salt excluders since unrestricted salt influx linked to transpirational water loss would rapidly lead to the accumulation of saturated salt in the leaves. This basic observation is well illustrated in work on the halophyte, *Leptochloa fusca* (Bhatti and Wienecke, 1984; Gorham, 1987; Jeschke *et al.*, 1995). Although it contains very effective salt glands, salt exclusion by these glands only makes a minor contribution to the regulation of the total leaf salt load, the rest depending on root exclusion and other mechanisms.

The interplay of apoplastic and symplasmic flows with the K^+/Na^+ selectivity of the various intracellular compartments on whole plant nutrient distribution and the role of wall suberization is well demonstrated in trans-cortical ion transport in roots. Suberization of the endodermal cell walls ensures that solute flux into the stele, as a prelude to xylem loading, is usually, but not exclusively (Garcia *et al.*, 1997; Steudle *et al.*, 1993; Yadav *et al.*, 1996; Yeo *et al.*, 1987) by a symplasmic pathway. Studies mainly on barley by Pitman and Jeschke and their co-workers (see Clarkson, 1988; Pitman, 1988) have also shown that in glycophytes the mechanism of xylem loading also discriminates in favour of K and against Na. Interestingly, X-ray microprobe analysis has suggested a gradient of K/Na across the root of *Lupinus* exposed to salinity suggesting replacement of vacuolar K^+ with Na^+ (van Steveninck *et al.*, 1982), a process limited by the total exchangeable K^+ available along the transport pathway. This strong discrimination in favour of K is difficult to reconcile with the many observations of Na accumulation in the leaves of halophytes even at quite low external salinities. A crucial observation was made by Stelter and Jeschke (1983) who found that in the halophyte, *Atriplex hortensis*, selectivity of xylem loading was reversed in favour of Na loading. This, with K^+ recirculation (Wolf and Jeschke, 1987; Wolf *et al.*, 1990), allows Na concentrations in the root symplasm to be quite low while ion fluxes are still sufficient to supply a major part of the leaf vacuolar solutes.

In the remainder of the chapter we will explore, briefly, two specific examples of how the ionic and K/Na selective characteristics of the symplasm/phloem continuum affect responses to salt and solute distribution.

8.5 Salts and root elongation growth

One of most striking examples of K/Na discrimination in saline conditions is found in root tips. Jeschke and Stelter (1976) showed that, even in barley roots exposed to K^+ -free media for many days and low concentrations of Na^+ salts, the terminal 1 mm or so

had a very high K/Na ratio. Further work on the halophytes *Atriplex hortensis* (Stelter and Jeschke, 1983) and *A. spongiosa* (Storey *et al.*, 1983 a, b) confirmed this finding. The shoot meristem of lettuce (*Lactuca sativa*) is also protected from salt-induced changes in ion content (Lazof and Lauchli, 1991). Radial ion distribution in barley roots was investigated by Pitman *et al.* (1980) and Yeo *et al.* (1977 a, b).

Intuitively it might be expected that external K^+ from the surrounding medium would be the immediate source of the nutrient to the root tip, but it is clear that this is not the case.

Jeschke, Pate and their colleagues have shown that adequate rates of K supply to root tips can be maintained by phloem transport from the main body of the plant even under salt stress and in the absence of external K^+ (Wolf and Jeschke, 1987). More recent work on solute deposition rates in cotton root tips has again confirmed that in the terminal 2 mm the rate of K^+ -deposition is maintained even at high (150 mol m^{-3}) NaCl levels, provided that the Ca^{++} concentration in the external medium is adequate (Zhong and Läuchli, 1994). Marschner *et al.* (1996) reached a similar conclusion.

The advent of the pressure probe (Tomos and Leigh, 1999) has allowed the turgor pressure of individual cells to be determined. The application of this technique to root tips has shown conclusively that turgor homeostasis is strongly maintained in the elongating cells over a wide range of conditions and that, especially in the proximal section of the elongating zone, K^+ is the dominant cation in both the cytoplasm and the vacuole (Pritchard *et al.*, 1996). While turgor homeostasis in the cells of the elongating zone is maintained over a range of external ionic conditions and under modest salt and osmotic stresses in some species, the growth rate can change dramatically. This further confirms that, although an adequate turgor is a prerequisite to growth, the elongation rate is modulated through changes in wall rheology. Several interesting issues arise. How is the turgor pressure maintained homeostatically? What is the source of the K^+ and how is its supply regulated. Is, indeed, the rate of K^+ uptake into these elongating cells part of the mechanism of the turgor-regulating process as it appears to be in bacteria? No definitive answers to these questions are currently available.

The use of a fluorescent probe has, however, allowed the real time unloading of the phloem contents in the root tips of *Arabidopsis* to be visualized (Oparka *et al.*, 1994; Wright *et al.*, 1996). The dye was observed to be rapidly translocated to the root tip followed by unloading into discrete concentric files. Other results on root tip sugar transport imply that preferential symplasmic transport takes place from the base of the protophloem through the cell files to meristem followed by basipetal loading of the more mature cells. In this context the arrangement and density of plasmodesmatal links suggest that these effectively extend phloem transport right through to the root meristem. These results, combined with the weight of evidence for a characteristic suite of symplasmic solutes, reinforce the tight link between cellular organization, specific plant functions and nutritional selectivity. Interestingly this hypothesis also suggests that symplasmic transport should be turgor regulated.

8.6 Intercellular variation in solute concentrations in leaves

The extension of the pressure probe methodology to allow the sampling of the solute content of individual vacuoles has added a new dimension to our understanding of solute relations in leaves and other issues. X-ray microprobe analysis provided indications that the solute contents of the vacuoles of the major cell types in leaves were not identical (Leigh and Tomos, 1993). Several studies have revealed large differences between epidermal and mesophyll cells of barley (Dietz *et al.*, 1992; Huang and Van Steveninck, 1989; Leigh and Storey, 1993; Leigh *et al.*, 1986; Williams *et al.*, 1993). Phosphate was found mainly in the mesophyll of barley, with only low concentrations in the epidermis, whereas Cl^- accumulated in the epidermis. Chloride also accumulated in epidermal layers of both sheaths and blades of sorghum leaves (Boursier and Läuchli, 1989). In barley, calcium accumulated in the epidermis rather than in the mesophyll.

In contrast, there is evidence that in the dicotyledons *Vicia faba* (Outlaw *et al.*, 1984), *Lycopersicon esculentum* (V. Cruz and R.G. Wyn Jones, unpublished) and cotton (Gorham *et al.*, unpublished) calcium and magnesium accumulated preferentially in the mesophyll and palisade cells, whereas phosphorus was accumulated in the epidermis. In *Lupinus luteus* van Steveninck *et al.* (1982) also found higher proportions of phosphorus in epidermal cells than in spongy mesophyll or palisade cells, and higher proportions of chloride in the spongy mesophyll than in the upper or lower epidermis. In *Aster tripolium*, a salt marsh plant, but not in the glycophyte *Commelina communis*, X-ray microanalysis revealed that Na^+ was excluded from stomatal guard cells when the plants were grown at high salinity (Perera *et al.*, 1977). Na^+ and Cl^- counts in the subsidiary and epidermal cells were higher on the upper than the lower surface of the leaf.

These observations have been confirmed and elaborated by Single Cell Sampling and Analysis (SiCSA). Importantly this sampling technique permits the simultaneous determination of cell turgor pressure, sap [vacuole] osmotic pressure and the concentrations of the major inorganic and organic solutes. In the leaves of barley grown with plenty of K^+ the vacuolar K^+ concentrations in the epidermal, mesophyll and bundle sheath cells are relatively uniform, but HPO_4^{2-} and Ca^{++} are non-uniformly distributed with high HPO_4^{2-} found in mesophyll vacuoles and Ca^{++} and Cl^- found in the epidermis.

Pertinent to the theme of this chapter is the effect of NaCl (100 mol m^{-3}) salinity on the vacuolar distribution of ions in barley leaf cells (Fricke *et al.*, 1996). As would be expected from the tissue analyses external salinity resulted in increased concentrations of Na^+ and Cl^- in the epidermal and mesophyll cells and a decline in K^+ and NO_3^- . The K^+ concentration declined more rapidly in mesophyll than in epidermal cells although in the latter the K^+ concentration ultimately declined to much lower levels. In epidermal cells the Cl^- level exceeded that of Na^+ but the reverse was observed in mesophyll cells that consistently had a lower total salt load. Fricke *et al.* (1994 a, c) have further shown differences in solute composition between the upper and lower

epidermis of barley, while Fricke *et al.* (1994 b) have reported spatial variations within the leaf epidermis.

These changes were accompanied by parallel changes in sap osmotic pressure. However these were not reflected in changes in turgor pressure that remain largely unaffected by external salinity. Two features emerge, firstly, turgor is more tightly regulated than either individual solute concentrations or osmotic potential and secondly turgor regulation can only have been achieved by a concomitant change in the extra-cellular water potential presumably by solute accumulation.

8.7 References

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CHAPTER 9

SALINITY, OSMOLYTES AND COMPATIBLE SOLUTES

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Abstract

In order to maintain low cytosolic Na^+ , and cytosolic K^+ concentrations within narrow limits (100 - 150 mM) across a broad range of external and vacuolar concentrations of NaCl, it is essential that other solutes be accumulated in the cytoplasm to keep this compartment in osmotic balance with the external medium and vacuole. Such solutes should be osmolytes that are non-toxic and “compatible” with cytoplasmic enzymes over wide concentration ranges. A number of such solutes have been identified, their pathways of synthesis elucidated, and progress has been in isolating genes encoding key enzymes of their biosynthetic pathways.

9.1 Introduction

In general, the enzymes of salt-tolerant plants are no more tolerant of deleterious inorganic ions, such as Na^+ , than are the enzymes of salt-sensitive plants (Flowers *et al.*, 1977). Plant adaptation to saline environments therefore requires the maintenance of low levels of Na^+ in the cytoplasm, achieved in part by accumulation of Na^+ in the vacuole (Flowers *et al.*, 1977). Eukaryotic cells are remarkably uniform in maintaining a fairly constant cytoplasmic K^+ concentration of between 100 and 150 mM (Wyn Jones *et al.*, 1977). In contrast to Na^+ , K^+ is essential for many processes in plants, including protein synthesis, membrane transport processes, generation of turgor, charge balance, and activation of certain enzymes (Leigh and Wyn Jones, 1984; Matoh *et al.*, 1988). The primary role of K^+ in the cytoplasm is for protein synthesis to maintain the association between tRNA and ribosomes during translation (Leigh and Wyn Jones, 1984). However, concentrations of K^+ exceeding 180 mM are inhibitory to protein synthesis because such concentrations promote dissociation of polysomes and

conformational changes in monosomes (Brady *et al.*, 1984). In general, K^+ is less inhibitory than Na^+ to enzyme function, but nevertheless both ions can be regarded as “strongly perturbing” to macromolecular structure and function at concentrations above 200 mM (Hochachka and Somero, 1984; Yancey, 1994). The sensitivity of protein synthesis to inorganic ions may set the boundaries for cell ionic strength (Leigh and Wyn Jones, 1984; Yancey, 1994).

Given the need to maintain low cytosolic Na^+ , and cytosolic K^+ concentrations within narrow limits (100 - 150 mM) across a broad range of external and vacuolar concentrations of NaCl, it is essential that other solutes be accumulated in the cytoplasm to keep this compartment in osmotic balance with the external medium and vacuole. Ideally, such solutes should be osmolytes that are non-toxic and “compatible” with cytoplasmic enzymes over wide concentration ranges (Wyn Jones *et al.*, 1977).

9.2 Compatible and non-compatible solutes

Figure 1 shows the Hofmeister series, representing a ranking of anions and cations in the order in which they influence macromolecular systems (Hochachka and Somero, 1984; Yancey, 1994). Ions to the left generally stabilize and enhance catalysis of macromolecular systems, while those to the right destabilize them. Those to the left promote “salting-out”, while those to the right promote “salting-in” of proteins. Salting-out and salting-in refer to the capacities of these ions to either decrease, or increase, respectively, the solubilities of proteins (Hochachka and Somero, 1984; Yancey, 1994). Note that Na^+ and K^+ rank in the middle of the series, but that K^+ is less destabilizing than Na^+ . Effects of the anions and cations are algebraically additive, leading to counteraction if opposing ion types in the series are paired (Yancey, 1994).

	← Stabilizing (Salting-Out)					Destabilizing (Salting-In) →				
Anions:	F^-	PO_4^{3-}	SO_4^{2-}	CH_3COO^-	Cl^-	Br^-	I^-	SCN^-		
Cations:	$N^+(CH_3)_4$	$N^+(CH_3)_2H_2$	N^+H_4	K^+	Na^+	Cs^+	Li^+	Mg^{2+}	Ca^{2+}	Ba^{2+}

Figure 1. The Hofmeister (lyotropic) series of inorganic ions. Anions and cations are ranked in the order (from left to right) of their increasing destabilizing effects on proteins. The ions that are the most stabilizing tend to promote “salting-out” of proteins (Hochachka and Somero, 1984; Yancey, 1994).

The perturbing ions to the right of Figure 1 are destabilizing to proteins because they readily enter the hydration sphere of proteins and bind to non-specific sites (e.g. charged protein groups), shifting the protein conformational equilibrium toward the unfolded state (Low, 1985; Yancey, 1994). The non-perturbing or “compatible” ions to the left of Figure 1 are stabilizing because they are preferentially excluded from the

protein hydration domain shell. This preferential exclusion causes proteins to fold more compactly by aggregation, or precipitation (salting-out) (Low, 1985; Yancey, 1994).

Compatible solutes are solutes which mimic the Hofmeister stabilizing ions of Figure 1 by being preferentially excluded from the protein surface and its immediate hydration sphere, and which stabilize folded protein structures, promote subunit assembly and tend to promote salting-out (Low, 1985; Yancey, 1994). Structures of several compatible solutes accumulated by plants are illustrated in Figure 2. These include amino acids, methylated onium compounds, and polyols (acyclic and cyclic polyhydric alcohols [cyclitols]). The compounds shown in Figure 2 will be the focus of this review. However, these represent only a small fraction of the known compatible solutes in the plant kingdom. A large number of other methylated onium compounds (not shown in Figure 2) have been identified in marine algae (Blunden and Gordon, 1986). The diversity of cyclic polyols found in higher plants is also far greater than depicted in Figure 2 (Popp and Smirnov, 1995, Chapter 6). Because of space constraints we will not consider carbohydrates (e.g. sucrose, trehalose, raffinose, stachyose and fructans) which may play important roles as compatible molecules during desiccation and freezing.

The methylated quaternary ammonium and tertiary ammonium compounds of Figure 2 can be seen to be structural homologs of the stabilizing Hofmeister anion, $\text{N}^+(\text{CH}_3)_4$. All of the compatible solutes shown in Figure 2 share the properties of being highly soluble in water, and are electrically neutral in the physiological pH range. Generally they are non-inhibitory to enzymes at high concentrations (Wyn Jones *et al.*, 1977), they can block the inhibition of enzyme activity caused by perturbing solutes (Yancey, 1994), and they elevate the denaturation temperatures of proteins (Low, 1985, Yancey, 1994). Many of the compatible solutes listed in Figure 2 have been shown to stimulate the growth of salt-sensitive bacteria (e.g. *Escherichia coli* and *Salmonella typhimurium*) in highly saline media ($> 0.6 \text{ M NaCl}$) (Strøm *et al.*, 1983; Hanson *et al.*, 1991; 1994 a). The growth stimulation properties of these compatible solutes is often referred to as “osmoprotection” (Strøm *et al.*, 1983; Hanson *et al.*, 1994 a). The term “osmoprotectant” is therefore commonly applied to many of these compounds (Hanson and Burnet, 1994; McNeil *et al.*, 1999; Nuccio *et al.*, 1999). When provided at low concentrations ($< 1 \text{ mM}$), compounds such as glycine betaine and proline are accumulated to near 1 M concentrations in the bacterial cytoplasm, facilitating Na^+ and Cl^- exclusion and replacement of K^+ and glutamate, which serve as the principal osmolytes in the absence of an exogenous supply of compatible solute (Cayley *et al.*, 1991, 1992). This replacement of K^+ /glutamate restores cell water content and growth (Cayley *et al.*, 1991, 1992).

Two explanations have been offered for the mechanism of compatible solute preferential exclusion from protein surfaces (Low, 1985; Yancey, 1994). First, many (but not all) of the solutes raise the surface tension of water, perhaps increasing the cohesive forces within the water structure; thus, they are proposed to be water structure-makers, enhancing water-water interactions (Low, 1985; Yancey, 1994).

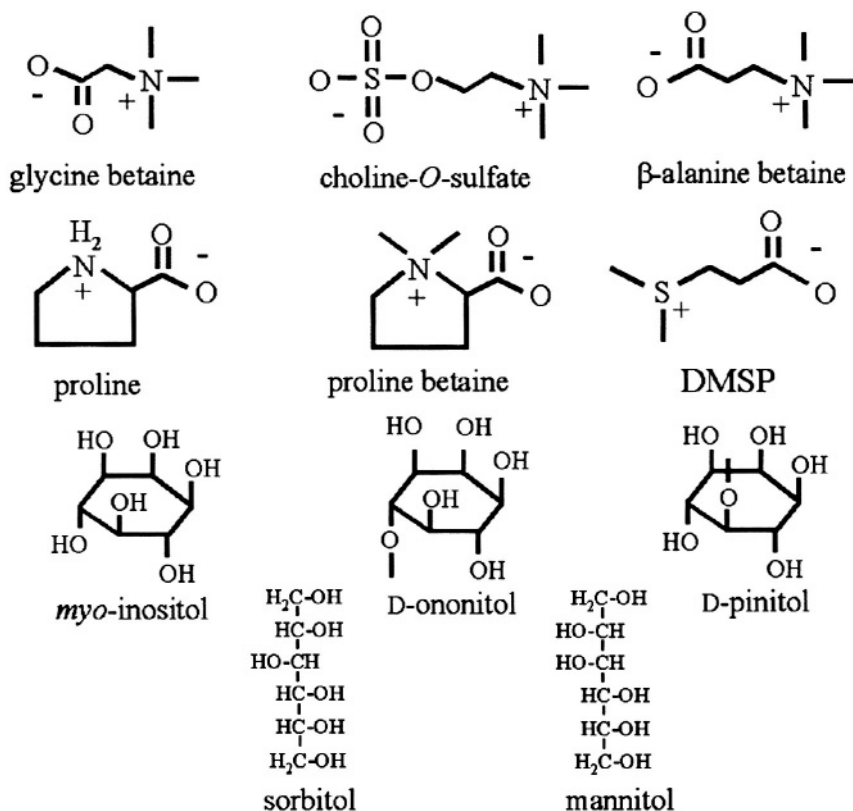


Figure 2. Structures of some compatible osmolytes found in plants. DMSP, 3-dimethylsulfoniopropionate.

They either enhance the existing structure of water or perhaps reorganize the bulk water to a lesser degree at their molecular surfaces than structure-breaking solutes (Low, 1985). Second, they may be sterically excluded from the protein surface (Low, 1985; Yancey, 1994). Because compatible solutes take up more space than a water molecule, they are less able to pack next to a protein than water. Bulky solutes will increase protein activity by “crowding” proteins into a smaller space (Yancey, 1994). Osmolytes with -OH groups that could form hydrogen bonds with proteins may have a poorer geometrical fit than water in the hydration lattice around the protein (Yancey, 1994).

In evolutionary terms the compatible solute accumulation strategy of adaptation to saline environments has key advantages over accumulation of cheaper, non-compatible, perturbing solutes, such as Na^+ and Cl^- , in the cytoplasm. Accumulation of perturbing solutes would require evolution of a large number of salt-tolerant enzymes, requiring substantial changes in protein amino acid composition to offset general Na^+ and Cl^- destabilization phenomena (Hochachka and Somero, 1984). Accumulation of a compatible solute for cytoplasmic osmoregulation would require far fewer changes, e.g.

modification of the regulatory mechanisms controlling biosynthesis or catabolism of a non-perturbing compound (Hochachka and Somero, 1984). There is therefore great interest in identifying genes encoding key enzymes of compatible solute synthesis and/or catabolism because these genes hold promise for improving crop resistance to osmotic stresses (Strøm *et al.*, 1983; Bohert and Jensen, 1996; Bohnert and Shen, 1999; Jain and Selvaraj, 1997; McNeil *et al.*, 1999; Nuccio *et al.*, 1999). In subsequent sections we will highlight recent progress in pathway characterization, gene identification and metabolic engineering of compatible solute accumulation in higher plants.

9.3 Pathways of synthesis and catabolism of compatible solutes found in plants

9.3.1 PROLINE

The imino acid proline is accumulated in the leaves of many halophytic plants from diverse families in response to salinity stress (Stewart and Lee, 1974; Treichel, 1975; Wyn Jones *et al.*, 1977; Briens and Larher, 1982; Stewart and Larher, 1980). Proline is derived primarily from glutamic acid in reactions catalyzed by Δ^1 -pyrroline-5-carboxylate synthetase (P5CS) and Δ^1 -pyrroline-5-carboxylate reductase (P5CR) (Figure 3) (Verma and Zhang, 1998). P5CS is a bifunctional enzyme that phosphorylates glutamate in the 5- (γ -) position to form γ -glutamyl phosphate, and then reduces this labile intermediate to glutamic-5-semialdehyde (GSA) (Hu *et al.*, 1992). GSA spontaneously cyclizes (with loss of water) to Δ^1 -pyrroline-5-carboxylate (P5C) (Figure 3). P5C is subsequently reduced to proline in the reaction catalyzed by P5CR (Verma and Zhang, 1998). P5CS is strongly feedback inhibited by proline (Verma and Zhang, 1998). Both enzymes of proline biosynthesis are localized in the cytosol. This contrasts with the proline catabolism pathway (catalyzed by proline dehydrogenase (PDH) and P5C dehydrogenase (P5CDH)), which is mitochondrial (reviewed by Hare and Cress (1997)) (Figure 3). Control of proline accumulation in response to salinity stress involves both increased synthesis and decreased proline oxidation, mediated (at least in some plant species) at the level of enhanced gene transcription of P5CS (Igarashi *et al.*, 1997; Yoshiba *et al.*, 1995; Strizhov *et al.*, 1997) and P5CR (Williamson and Slocum, 1992; Verbruggen *et al.*, 1993), and concomitant down-regulation of PDH gene expression (Kiyosue *et al.*, 1996; Peng *et al.*, 1996; Yoshiba *et al.*, 1997). Whereas PDH is normally induced by proline, this induction does not occur under osmotic stress (Peng *et al.*, 1996). It is noteworthy, however, that in tomato cell cultures adapted to salinity stress (15 g NaCl/L) a 30-fold increase in proline level occurs without any notable change in P5CS mRNA abundance (Fujita *et al.*, 1998). Ketchum *et al.* (1991) conclude from inhibitor studies with suspension cells of the halophytic grass, *Distichlis spicata*, that mRNA translation but not transcription is necessary for salinity stress induced proline accumulation, implying a post-transcriptional control mechanism(s).

Over-expression of P5CS in tobacco leads to proline accumulation and increased resistance to salinity stress (Kishor *et al.*, 1995). It is not clear whether the enhanced salt resistance of these transgenic plants can be ascribed solely to proline acting as a cytoplasmic compatible solute. Proline can also function as a hydroxyl radical scaven-

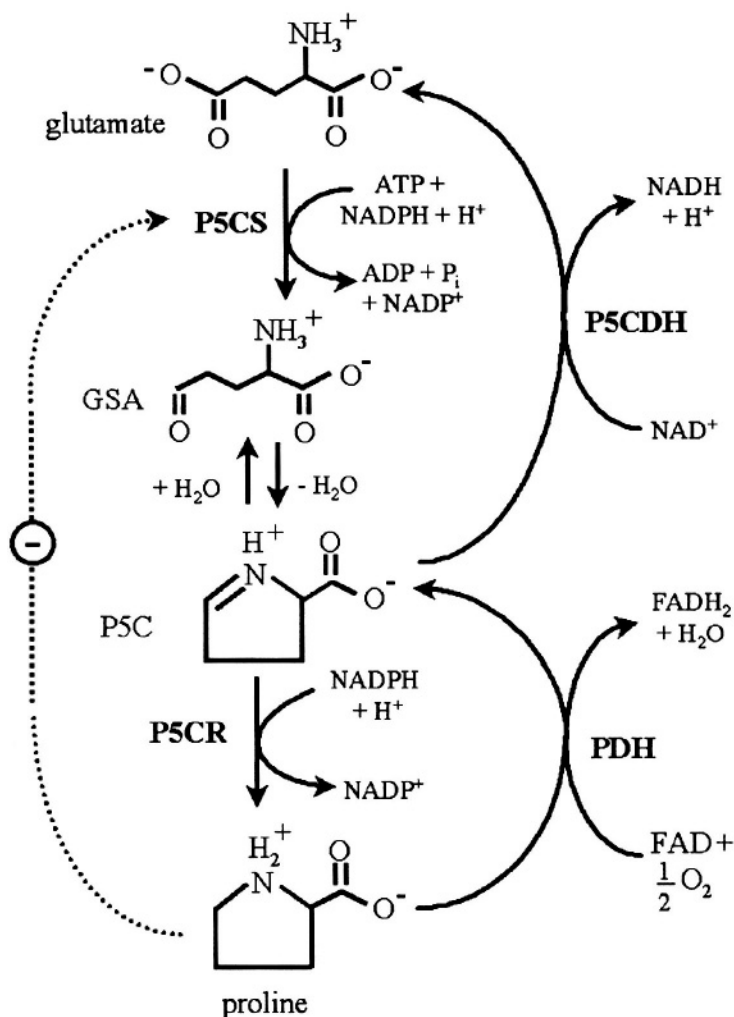


Figure 3. Pathways of synthesis and oxidation of proline in plants. GSA, glutamic-5-semialdehyde; P5C, Δ¹-pyrroline-5-carboxylate; P5CDH, Δ¹-pyrroline-5-carboxylate dehydrogenase; P5CR, Δ¹-pyrroline-5-carboxylate reductase; P5CS, Δ¹-pyrroline-5-carboxylate synthetase. The dotted line denotes feedback inhibition of P5CS by proline.

ger (Smirnoff and Cumbes, 1989), and may play a role in the transport of reducing equivalents between cells and intracellular compartments (i.e. between cytoplasm and mitochondrion) (Hare and Cress, 1997).

9.3.2 GLYCINE BETAINE

Glycine betaine is widely distributed in the plant kingdom (Wyn Jones and Storey, 1981). It is accumulated to osmotically significant levels by many halophytic higher plants, including members of the Chenopodiaceae and salt-tolerant species of the Poaceae (Rhodes and Hanson, 1993). In chenopods, glycine betaine is synthesized from choline in reactions catalyzed by ferredoxin (Fd)-dependent choline monooxygenase (CMO) and betaine aldehyde dehydrogenase (BADH) (Figure 4A). Both CMO and BADH are predominantly localized in the chloroplast stroma of chenopods (Weigel *et al.*, 1986, 1988; Brouquisse *et al.*, 1989). Glycine betaine is also localized in the chloroplasts of salinized spinach leaves, where it provides osmotic adjustment (Robinson and Jones, 1986), and may play a key role in protection of the photosynthetic apparatus, particularly photosystem II (Murata *et al.*, 1992; Papageorgiou *et al.*, 1991; Homann, 1992; Williams and Gounaris, 1992; Mohanty *et al.*, 1993; Papageorgiou and Murata, 1995).

Both CMO (Burnet *et al.*, 1995) and BADH (Weretilnyk and Hanson, 1989) have been purified to homogeneity from spinach. cDNAs encoding BADH have been isolated from spinach (Weretilnyk and Hanson, 1990; Rathinasabapathi *et al.*, 1994) and sugar beet (McCue and Hanson, 1992 b). A cDNA encoding CMO has also been cloned from spinach (Rathinasabapathi *et al.*, 1997). The cDNA sequence confirms that CMO is an Fe-S protein and that CMO has a typical chloroplast transit peptide sequence.

BADH may be a relatively non-specific aldehyde dehydrogenase, acting on other aldehyde substrates in addition to betaine aldehyde (Trossat *et al.*, 1997). Thus, BADH will utilize 3-dimethylsulfoniopropionaldehyde, an intermediate in 3-dimethylsulfoniopropionate (DMSP) synthesis [see 9.3.6 below] and aldehydes (3-aminopropionaldehyde and 4-aminobutyraldehyde) involved in polyamine metabolism (Trossat *et al.*, 1997). This may explain the occurrence of BADH in plants that do not accumulate glycine betaine (Ishitani *et al.*, 1993; Weretilnyk *et al.*, 1989), and in organs of glycine betaine-accumulating plants which do not contain glycine betaine (e.g. roots of cereals) (Ishitani *et al.*, 1995).

Salinity stress leads to two- to three-fold increases of CMO and BADH gene expression, and concomitant increases in enzyme level in spinach, sugar beet, and amaranth (Weretilnyk and Hanson, 1989, 1990; Hanson *et al.*, 1995; Russell *et al.*, 1998). Absciscic acid (ABA) is implicated in the induction of BADH in cereals (Ishitani *et al.*, 1985). The signal causing induction of BADH in sugar beet does not appear to be either turgor reduction, NaCl, or ABA, but rather an unidentified biochemical signal translocated from roots to leaves in salinized plants (McCue and Hanson, 1992 b). Note that unlike proline, glycine betaine is not actively catabolized, so that control of glycine betaine concentration resides primarily at the level of synthesis rate and the rate of pool dilution with growth (Rhodes and Hanson, 1993).

In both maize and sorghum a number of naturally occurring glycine betaine-deficient inbred lines have been identified (Brunk *et al.*, 1989; Grote *et al.*, 1994). Glycine betaine-deficient maize lines lack the ability to accumulate glycine betaine in leaf tissue in response to either salinity stress or water deficits, due to a single recessive gene

(*bet1*) (Yang *et al.*, 1995). Homozygous *bet1/bet1* maize lines are more salt-sensitive than near-isogenic homozygous *Bet1/Bet1* lines (Saneoka *et al.*, 1995), and exhibit greater membrane injury and damage to photosystem II in response to heat stress

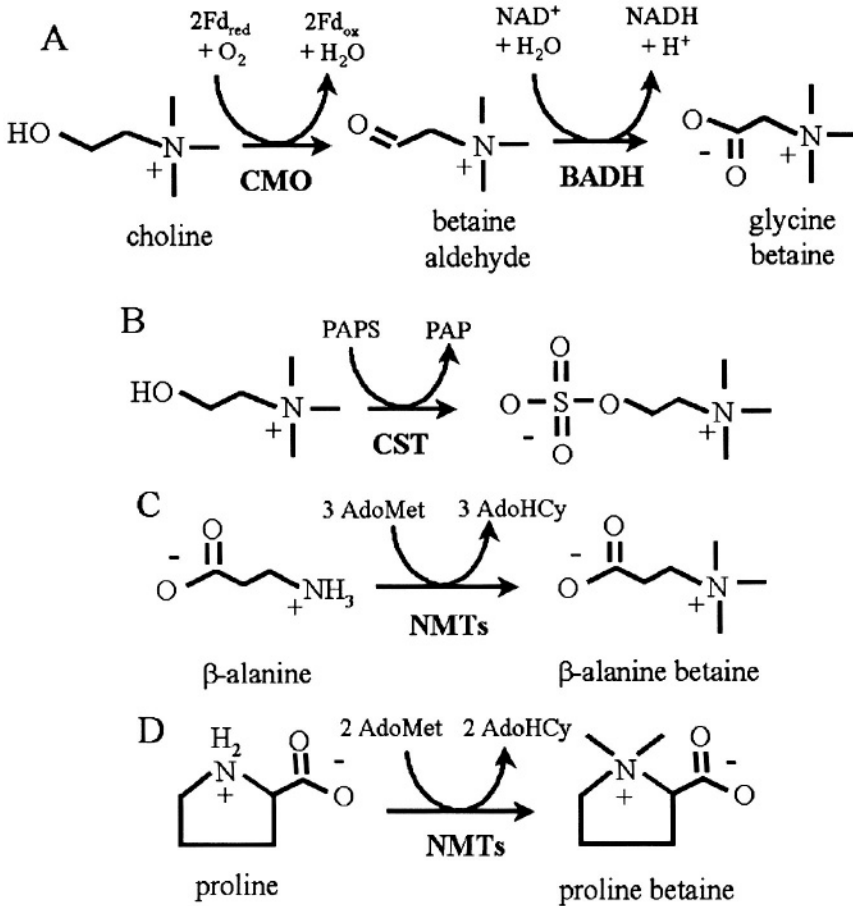


Figure 4. Pathways of synthesis of glycine betaine (A), choline-O-sulfate (B), β -alanine betaine (C), and proline betaine (D). AdoHCy, *S*-adenosyl-homocysteine; AdoMet, *S*-adenosylmethionine; BADH, betaine aldehyde dehydrogenase; CMO, choline monooxygenase; CST, choline sulfotransferase; Fd, ferredoxin; NMTs, *N*-methyltransferases; PAP, 3'-phosphoadenosine-5'-phosphate; PAPS, 3'-phosphoadenosine-5'-phosphosulfate.

(Yang *et al.*, 1996). Glycine betaine-deficient maize lines lack the capacity to metabolize ^{14}C -choline to ^{14}C -glycine betaine, but are unimpaired in their ability to metabolize $^2\text{H}_3$ -betaine aldehyde to $^2\text{H}_3$ -glycine betaine, suggesting a lesion at the CMO step [or equivalent choline oxidizing activity] in the glycine betaine biosynthetic pathway (Lerma *et al.*, 1991). Because BADH appears to be localized in peroxisomes of monocotyledonous plants (Nakamura *et al.*, 1997), this raises the question of whether the subcellular localization [and mechanism] of choline oxidation is the same

in grasses as in chenopods. Whereas chenopods derive choline destined for oxidation to glycine betaine predominantly from phosphocholine, via the action of a phosphocholine phosphatase (Hanson and Rhodes, 1981; Hanson *et al.*, 1995), grasses obtain choline primarily from phosphatidylcholine (Hitz *et al.*, 1981), presumably via the action of phospholipase D (Rhodes and Hanson, 1993).

In bacteria such as *Escherichia coli*, choline is an osmoprotectant only because it is oxidized to glycine betaine. The choline-glycine betaine pathway of *E. coli* consists of several genes encoding the functions of choline transport (*betT*), choline dehydrogenase (*betA*), betaine aldehyde dehydrogenase (*betB*), and a regulatory locus (*betI*) encoding a repressor protein (Lamark *et al.*, 1991, 1996). The entire *E. coli bet* gene cluster has been introduced into the freshwater cyanobacterium, *Synechococcus*, and this confers glycine betaine accumulation (60 to 80 mM) and increased salt tolerance, due in part to stabilization of photosystems I and II (Nomura *et al.*, 1995), and protection of ribulose 1,5-bisphosphate carboxylase/oxygenase from inactivation (Nomura *et al.*, 1998). However, this increased salt tolerance appears to depend on the presence of an exogenous supply of choline (Nomura *et al.*, 1995).

Because tobacco, rice and *Arabidopsis* are non-glycine betaine accumulators, these have been the organisms of choice for preliminary metabolic engineering experiments to test whether introduction of a capacity to synthesize glycine betaine influences higher plant salt tolerance (Hayashi and Murata, 1998; Takabe *et al.*, 1998; Nuccio *et al.*, 1998; McNeil *et al.*, 1999). Transgenic tobacco plants expressing the *E. coli betA* gene encoding choline dehydrogenase, putatively capable of oxidizing both choline and betaine aldehyde, are reported to have increased salt tolerance (Lilius *et al.*, 1996), but because glycine betaine was not measured in these plants it is uncertain whether the salt tolerance phenotype can be attributed to glycine betaine accumulation. Transgenic rice plants expressing the *E. coli betA* gene are reported to accumulate up to 5 $\mu\text{mol/g}$ fresh weight of glycine betaine associated with increased salt tolerance (Takabe *et al.*, 1998). Introduction of the choline oxidase (*codA*) gene from *Arthrobacter* spp. into *Arabidopsis thaliana*, targeted to the chloroplast, leads to modest glycine betaine accumulation (1 $\mu\text{mol/g}$ fresh weight) [approx. 50 mM if assumed to be confined solely to the chloroplast], and enhanced salt and cold tolerance associated with protection of photosystem II (Hayashi *et al.*, 1997; Hayashi and Murata, 1998). The *codA* gene also appears to confer increased heat and light stress tolerance in *Arabidopsis* (Alia *et al.*, 1998, 1999). The *codA* gene has also been expressed in either the chloroplast or cytosol of rice (Sakamoto *et al.*, 1998). Chloroplastic expression appears to confer greater tolerance to photoinhibition under salinity and low temperature stress (Sakamoto *et al.*, 1998). However, expression of the cDNA encoding spinach CMO in the chloroplasts of tobacco leads to glycine betaine accumulation to levels of only a few percent of those found in natural glycine betaine accumulators (Nuccio *et al.*, 1998). Detailed analyses of choline metabolism in these transgenic tobacco plants suggest that the supply of choline may be limiting (Nuccio *et al.*, 1998, 1999). In tobacco, cytosolic choline is channeled almost exclusively into phosphatidylcholine via phosphocholine, making it difficult to divert choline to glycine betaine (Nuccio *et al.*, 1998). Glycine betaine accumulation places large demands on the synthesis of choline moieties, and on methyl

groups required to generate choline (Hanson *et al.*, 1995). The capacity to accumulate glycine betaine may depend on special adaptations in choline and methyl group biogenesis that are not expressed in non-accumulators (Hanson *et al.*, 1995; McNeil *et al.*, 1999). In tobacco, and other non-glycine betaine accumulators, choline synthesis may be constrained at the first step in methylation of phosphoethanolamine en route to phosphocholine, catalyzed by phosphoethanolamine *N*-methyltransferase (Nuccio *et al.*, 1998, 1999).

9.3.3 CHOLINE-*O*-SULFATE

All members of the Plumbaginaceae accumulate choline-*O*-sulfate (Hanson *et al.*, 1994 a). This compound is synthesized from choline by a salt stress-inducible choline sulfotransferase (Rivoal and Hanson, 1994) (Figure 4B). Salt glands of members of the Plumbaginaceae can excrete chloride but not sulfate; conjugation of sulfate with choline may therefore be a mechanism of sulfate detoxification, converting a normally destabilizing anion to a compatible solute (Hanson *et al.*, 1994 a). Consistent with this, accumulation of choline-*O*-sulfate is enhanced when Cl^- is replaced with SO_4^{2-} in the salinization medium (Hanson *et al.*, 1991). Because choline-*O*-sulfate synthesis competes with glycine betaine synthesis for available choline, this may have contributed to the evolution of alternative betaine biosynthesis pathways (**β -alanine** betaine or proline betaine) which draw on substrates other than choline (Hanson *et al.*, 1994 a).

9.3.4 **β -ALANINE** BETAINE

β -Alanine betaine is restricted to species of the Plumbaginaceae (Hanson *et al.*, 1994 a). It is synthesized by direct *N*-methylation of the amino acid **β -alanine**, catalyzed by at least two *S*-adenosylmethionine (AdoMet)-dependent *N*-methyltransferases (Hanson *et al.*, 1991, 1994 a) (Figure 4C). Unlike glycine betaine, **β -alanine** betaine synthesis does not require O_2 (Hanson *et al.*, 1994 a). This pathway may therefore represent an adaptation to anoxic saline environments (Hanson *et al.*, 1994 a). The **β -alanine** betaine biosynthesis pathway is active in both leaves and roots of **β -alanine** betaine-accumulating *Limonium* species (Hanson *et al.*, 1991). However, the precise origin of **β -alanine** in the Plumbaginaceae is not known.

9.3.5 PROLINE BETAINE AND HYDROXYPROLINE BETAINES

Proline betaine is accumulated by many higher plant species from diverse families (Wyn Jones and Storey, 1981; Hanson *et al.*, 1994 a; Gorham, 1995; Nolte *et al.*, 1997). The synthesis of proline betaine is thought to involve *N*-methylation of proline, via the intermediate *N*-methylproline (hygric acid) (Essery *et al.*, 1962), presumably catalyzed by AdoMet-dependent methyltransferases (Figure 4D) (Hanson *et al.*, 1994 a). However, these enzymes have not yet been characterized. Hydroxyproline betaine often accumulates with proline betaine (Hanson *et al.*, 1994 a; Nolte *et al.*,

1997). The proline and/or *N*-methylproline hydroxylase(s) putatively involved in hydroxyproline betaine synthesis have not yet been identified (Hanson *et al.*, 1994 a).

Proline betaine and hydroxyproline betaine accumulate at the expense of free proline (Hanson *et al.*, 1994 a; Nolte *et al.*, 1997). Because proline betaine is a more potent osmoprotectant than proline, this may confer increased osmotic stress resistance (Hanson *et al.*, 1994 a). Synthesis of proline betaine would be expected to both alleviate P5CS from feedback regulation by proline, and restrict proline oxidation and hence compatible solute catabolism, perhaps contributing to maintenance of a greater compatible solute concentration (Samaras *et al.*, 1995).

9.3.6 DIMETHYLSULFONIOPROPIONATE

The sulfonium analog of β -alanine betaine, 3-dimethylsulfoniopropionate (DMSP), is synthesized and accumulated to osmotically significant levels by many marine algae (see e.g. Blunden and Gordon, 1986; Dickson and Kirst, 1986), and by a few higher plant species from diverse families; Asteraceae (e.g. *Wollastonia biflora*), and Poaceae (some species of *Spartina* and *Saccharum*) (Paquet *et al.*, 1994; James *et al.*, 1995). DMSP is as effective as glycine betaine as an osmoprotectant for *E. coli* (Paquet *et al.*, 1994), and is noted to be a potent cryoprotectant (Nishiguchi and Somero, 1992; Karsten *et al.*, 1996).

In *Wollastonia*, DMSP is synthesized by methylation of methionine to *S*-methylmethionine (SMM) in the cytosol (Hanson *et al.* 1994 b; James *et al.* 1995 a, b), transport of SMM into the chloroplast, transamination or deamination and decarboxylation of SMM to DMSP-aldehyde (James *et al.*, 1995 b), and oxidation of the aldehyde to DMSP in the chloroplast (Trossat *et al.*, 1996, 1997) (Figure 5). The first enzyme of the pathway, AdoMet:L-methionine *S*-methyltransferase (MMT) has been purified from *Wollastonia* leaves (James *et al.*, 1995 a), and its cDNA cloned (Bourgis *et al.*, 1999). The final step in the pathway is catalyzed by a chloroplast localized DMSP-aldehyde dehydrogenase (DADH), which may be identical to the BADH involved in glycine betaine synthesis (Trossat *et al.*, 1997; Vojtechová *et al.*, 1997). The enzyme(s) responsible for metabolism of SMM to DMSP-aldehyde in *Wollastonia* is not yet known. It is proposed that SMM is converted to DMSP-aldehyde via a specialized transaminase-decarboxylase (Rhodes *et al.*, 1997).

The pathway of DMSP synthesis in the salt marsh grass *Spartina alterniflora* is similar to that in *W. biflora* except with respect to the **SMM \rightarrow DMSP-aldehyde** steps in the pathway (Figure 5). In *Spartina*, radiolabeling experiments with ³⁵S-methionine and ³⁵S-SMM show that DMSP-amine behaves as an intermediate, implicating SMM decarboxylation as the first step in the conversion of SMM to DMSP-aldehyde (Kocsis *et al.*, 1998). DMSP-amine may then be metabolized to the aldehyde via an amine oxidase, but this enzyme has not been characterized.

The pathway of DMSP synthesis in higher plants is completely different from that found in marine algae (Gage *et al.*, 1997). In marine algae methionine is first converted to the 2-keto acid, 4-methylthio-2-oxobutyrates (MTOB), via the action of

either methionine:2-oxoglutarate aminotransferase or methionine oxidase (Gage *et al.*, 1997; Summers *et al.*, 1998) (Figure 5). MTOB is then reduced to 4-methylthio-2-hydroxybutyrate (MTHB) by an NADPH-dependent reductase (Summers *et al.*, 1998). MTHB is subsequently *S*-methylated to 4-dimethylsulfonio-2-hydroxybutyrate (DMSHB) which is then oxidatively decarboxylated to DMSP (Gage *et al.*, 1997; Summers *et al.*, 1998) (Figure 5). The latter enzyme has not been identified.

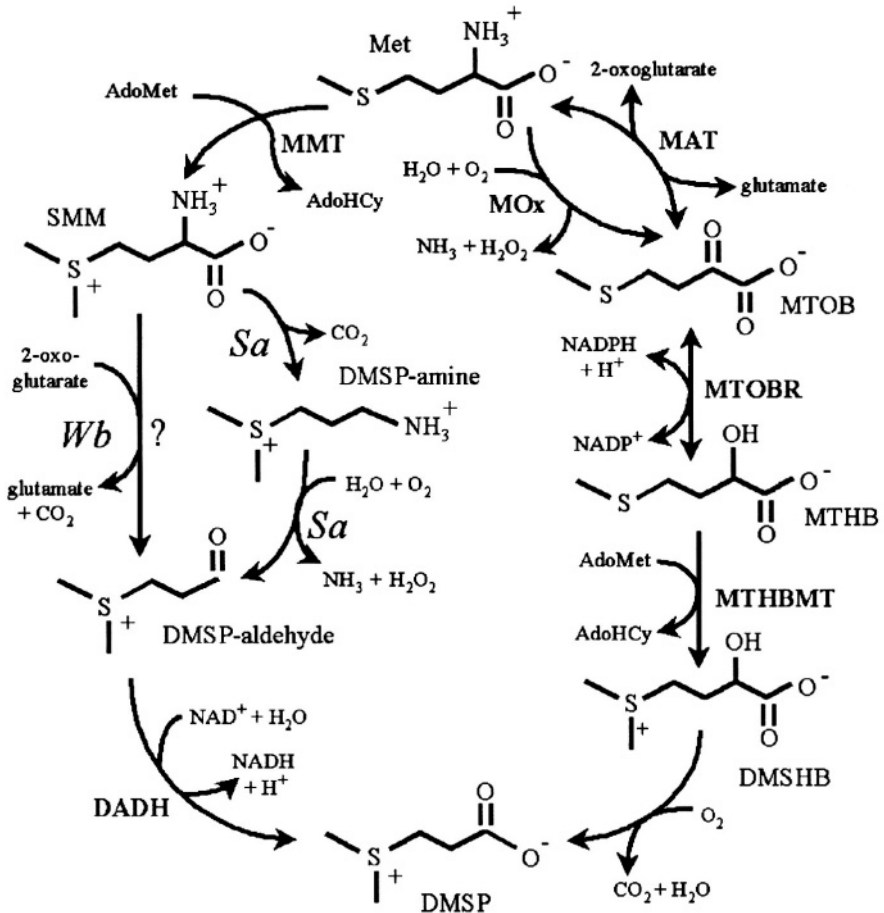


Figure 5. Pathways of synthesis of 3-dimethylsulfoniopropionate (DMSP) in higher plants (left) and marine algae (right). For the higher plant pathway *Wb* denotes the pathway found in *Wollastonia biflora* (Asteraceae), and *Sa* the pathway in *Spartina alterniflora* (Poaceae). The question mark adjacent to *Wb* indicates that the precise mechanism of conversion of SMM to DMSP-aldehyde in *Wollastonia* is not known; it may involve transamination and decarboxylation. AdoHCy, *S*-adenosylhomocysteine; AdoMet, *S*-adenosyl-methionine; DADH, DMSP-aldehyde dehydrogenase; DMSHB, 4-dimethylsulfonio-2-hydroxybutyrate; DMSP-amine, 3-dimethylsulfoniopropylamine; DMSP-aldehyde, 3-dimethylsulfonio-propionaldehyde; MAT, methionine:2-oxoglutarate aminotransferase; Met, methionine; MMT, AdoMet:methionine *S*-methyltransferase; MOx, methionine oxidase; MTHB, 4-methylthio-2-hydroxybutyrate; MTHBMT, AdoMet:MTHB *S*-methyltransferase; MTOB, 4-methylthio-2-oxobutyrate; MTOBR, MTOB reductase; SMM, *S*-methylmethionine.

Because DMSP does not contain N, the accumulation of DMSP as a compatible solute could represent a special adaptation to N-limiting environments. N-deficiency increases DMSP levels in *Wollastonia* (Hanson *et al.*, 1994 b) and marine algae (Gage *et al.*, 1997; Dickson and Kirst, 1986).

9.3.7 POLYOLS

Like DMSP, polyhydric alcohols (polyols) also lack N, and so could represent important compatible solutes where synthesis of nitrogenous solutes (e.g. proline and/or betaines) is limited by N availability. The acyclic polyols, sorbitol and mannitol are accumulated by many marine algae, lower plants and species from diverse higher plant families, including certain halophytes (Ahmad *et al.*, 1979; Briens and Larher, 1982; 1983; Popp and Smirnov, 1995). Sorbitol is accumulated to high levels (up to 70 mM on a tissue water basis) by members of the Plantaginaceae in response to salinity stress, while mannitol can reach concentrations as high as 200 mM on a tissue water basis in leaf and stem tissues of the mangrove species *Laguncularia racemosa* (Popp and Smirnov, 1995).

Figure 6 shows the pathways of synthesis of sorbitol, mannitol and the cyclic polyols (cyclitols), D-ononitol and D-pinitol. Note that sorbitol and mannitol draw directly from the glucose-6-phosphate and fructose-6-phosphate pools, and that sorbitol synthesis potentially competes with *myo*-inositol synthesis, and hence the synthesis of D-ononitol and D-pinitol. The latter cyclic polyols are accumulated by many legumes in response to water deficits and salinity stress (Popp and Smirnov, 1995). D-Pinitol is also accumulated by the halophytic facultative crassulacean acid metabolism plant, *Mesembryanthemum crystallinum*, in response to salinization (Bohnert *et al.*, 1995; Bohnert and Jensen, 1996).

In the Rosaceae and Apiaceae, sorbitol and mannitol, respectively, are utilized as phloem translocated photoassimilates (Locy, 1994; Popp and Smirnov, 1995). These polyols are actively catabolized in non-photosynthetic sink tissues by **NAD⁺-dependent** sorbitol and mannitol dehydrogenases (Locy, 1994; Williamson *et al.*, 1995). The mannitol dehydrogenase of celery has been cloned and has been shown to be repressed by salinity stress (Williamson *et al.*, 1995). In celery plants mannitol dehydrogenase activity and mannitol pool size are inversely correlated, suggesting that regulation of catabolism of mannitol is a major determinant of the tissue concentration of this compatible solute (Williamson *et al.*, 1995). However, little is known about the regulation of sorbitol and mannitol catabolism in members of the Plantaginaceae and mangroves that accumulate high levels of these solutes in response to salinity stress (Locy, 1994; Popp and Smirnov, 1995). The cyclic polyols, D-ononitol and D-pinitol, appear to be less rapidly catabolized (Popp and Smirnov, 1995), and control of tissue concentrations may therefore be primarily determined by synthesis rate.

In members of the Apiaceae (e.g. celery and celeriac), mannitol is synthesized from fructose-6-phosphate via mannose-6-phosphate via the action of mannose-6-phosphate isomerase and an NADPH-dependent mannose-6-phosphate reductase (M6PR) (Figure 6). The gene encoding M6PR has been cloned from celery, and shows

considerable sequence similarity to the sorbitol-6-phosphate dehydrogenase of apple (Everard *et al.*, 1997). M6PR is expressed in green tissues and is under tight transcriptional regulation during leaf development (Everard *et al.*, 1997).

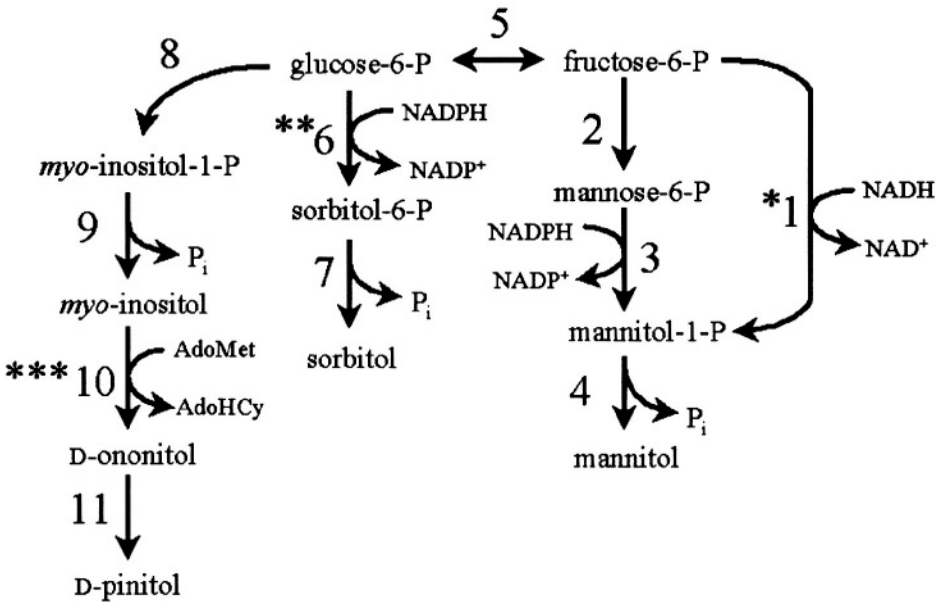


Figure 6. Pathways of synthesis of the acyclic polyols, mannitol and sorbitol, and the cyclic polyols, *myo*-inositol, D-ononitol, and D-pinitol. * Denotes genes used in metabolic engineering experiments with tobacco (see text for details). Enzymes (and genes) are as follows:

1. Mannitol-1-phosphate dehydrogenase,
* *mtlD* (*Escherichia coli*)
2. Mannose-6-phosphate isomerase.
3. Mannose-6-phosphate reductase.
4. Mannitol-1-phosphate phosphatase.
5. Hexose-phosphate isomerase.
6. Sorbitol-6-phosphate dehydrogenase [aldose-6-phosphate reductase (NADPH)],
** *S6pdh* (*Malus domestica*).
7. Sorbitol-6-phosphate phosphatase.
8. Glucose-6-phosphate cycloaldolase [*myo*-inositol-1-phosphate synthase].
9. *Myo*-inositol-1-phosphate phosphatase [*inositol* monophosphatase].
10. *Myo*-inositol-*O*-methyltransferase,
*** *Imt1* (*Mesembryanthemum crystallinum*).
11. Ononitol epimerase.

Tobacco has served as an excellent model system to explore the potential contribution of the acyclic and cyclic polyols. Wild-type tobacco lacks mannitol, sorbitol, D-ononitol and D-pinitol, but contains *myo*-inositol (Sheveleva *et al.*, 1997, 1998). Tobacco has been engineered to accumulate mannitol by introducing the mannitol-1-phosphate dehydrogenase gene (*mtlD*) from *Escherichia coli* (Tarczynski *et al.*, 1992, 1993). This

enzyme converts fructose-6-phosphate to mannitol-1-phosphate, which is then cleaved to mannitol, presumably by a constitutive, non-specific sugar alcohol-phosphate phosphatase (Figure 6). Constitutive production of mannitol in tobacco has been achieved by expressing *mtlD* in either the cytosol (Tarczynski *et al.*, 1992, 1993) or chloroplast (Shen *et al.*, 1997 a, b); both result in increased salt tolerance. Expression of *mtlD* in the chloroplast results in accumulation of mannitol to concentrations of up to 100 mM in tobacco chloroplasts (Shen *et al.*, 1997 b). Mannitol may function not only as a compatible solute, but also as a hydroxyl radical scavenger in this compartment (Smirnov and Cumbes, 1989; Shen *et al.*, 1997 b).

In apple, sorbitol is derived from glucose-6-phosphate via the catalytic action of an NADPH-dependent sorbitol-6-phosphate dehydrogenase (Tao *et al.*, 1995), and sorbitol-6-phosphate phosphatase (Popp and Smirnov, 1995) (Figure 6). Constitutive expression of the apple sorbitol-6-phosphate dehydrogenase in tobacco, leads to necrotic lesions in proportion to the size of the sorbitol pool accumulated [up to 130 mM on a whole tissue water basis] (Sheveleva *et al.*, 1998). Again, as with mannitol, it is presumed that sorbitol-6-phosphate is catabolized to sorbitol by a constitutive non-specific sugar alcohol-phosphate phosphatase in these transgenic tobacco plants (Sheveleva *et al.*, 1998). The autotoxicity of sorbitol accumulation in tobacco may be the result of depletion of *myo*-inositol (Sheveleva *et al.*, 1998), although toxic accumulation of sorbitol-6-phosphate cannot yet be ruled out. Because of the growth inhibition caused by overproduction of sorbitol in transgenic tobacco, it has not been possible to accurately assess the contribution of this solute to salt tolerance of tobacco (Sheveleva *et al.*, 1998).

Myo-inositol is derived from glucose-6-phosphate via the action of glucose-6-phosphate cycloaldolase [*myo*-onositol-1-phosphate synthase] and *myo*-inositol-1-phosphate phosphatase (Figure 6). D-Ononitol is then formed by *O*-methylation of *myo*-inositol, catalyzed by an AdoMet-dependent *myo*-inositol *O*-methyltransferase (Vernon and Bohnert, 1992). D-Pinitol is derived from D-ononitol via the action of an ononitol epimerase (Bohnert and Jensen, 1996) (Figure 6). In *M. crystallinum* the entire pathway is salinity stress inducible (Vernon and Bohnert, 1992; Bohnert and Jensen, 1996). *Myo*-inositol may not only serve as a substrate for the production of compatible solutes but also as a leaf-to-root signal that promotes sodium uptake in *M. crystallinum* (Nelson *et al.*, 1999). Note that wild-type tobacco has the capacity to synthesize and accumulate *myo*-inositol in response to salinity and drought stress, but lacks the terminal methyltransferase and epimerase of the pathway (Sheveleva *et al.*, 1997). Sheveleva *et al.* (1997) have constitutively expressed the *M. crystallinum* gene encoding *myo*-inositol-*O*-methyltransferase in tobacco. This confers stress-induced D-ononitol accumulation [up to 35 mM on a whole tissue water basis] and increased salt tolerance (Sheveleva *et al.*, 1997). Unlike constitutive sorbitol accumulation, stress-inducible D-ononitol accumulation does not result in necrotic lesions (Sheveleva *et al.*, 1997).

9.4 Evidence for cytoplasmic localization of compatible solutes

Central to the compatible solute hypothesis is that these solutes are accumulated in the cytosol and/or cytoplasmic organelles such as chloroplasts. Approaches to testing this fundamental premise of this hypothesis have been to isolate intact vacuoles and chloroplasts from salinized plant tissues, demonstrating that the ratios of compatible solutes to inorganic ions in these compartments are substantially different from those of whole tissue extracts (Leigh *et al.*, 1981; Wyn Jones *et al.*, 1977; Robinson and Jones, 1986). However, considerable technical difficulties are associated with correcting for solute leakage from organelles during aqueous extraction (see e.g. Leigh *et al.*, 1981; Robinson and Jones, 1986).

Using organelle isolation methods evidence for a predominantly cytoplasmic (extra-vacuolar) localization of proline has been obtained for red beet cells (Leigh *et al.*, 1981) and *Distichlis spicata* cell cultures (Ketchum *et al.*, 1991). The cytoplasmic concentration of proline in 200 mM NaCl-grown *Distichlis* cells has been determined to be within the range 237 - 311 mM; approximately 10-times the concentration in the cell as a whole (Ketchum *et al.*, 1991).

Robinson and Jones (1986) have determined that the glycine betaine concentration of chloroplasts isolated from leaves of spinach plants salinized to 200 mM NaCl, can approach 300 mM, contributing at least one-third of the total osmotic potential of the chloroplast. This is consistent with other investigations of the compartmentation of glycine betaine in chenopods which have demonstrated that this solute is extra-vacuolar (Wyn Jones *et al.*, 1977; Hall *et al.*, 1978; Leigh *et al.*, 1981; Matoh *et al.*, 1987) or chloroplastic (Schröppel-Meier and Kaiser, 1988; Genard *et al.*, 1991). It is not known whether glycine betaine is localized in chloroplasts of grasses which accumulate this solute. The intracellular localizations of choline-*O*-sulfate, **β -alanine** betaine, proline betaine and hydroxyproline betaine in the Plumbaginaceae have not been investigated. However, there is strong circumstantial evidence that these solutes have replaced the function of glycine betaine as cytoplasmic compatible solutes. Thus, the total quaternary ammonium compound levels of contrasting glycine betaine and **β -alanine** betaine accumulating *Limonium* species are both tightly correlated with total solute potential (at 100% relative water content) in leaf tissues of plants grown in nutrient solutions containing 0 - 600 mM NaCl (Hanson *et al.*, 1991).

Consistent with the chloroplastic localization of the terminal enzymes of the DMSP synthesis pathway in *Wallastonia biflora*, DMSP is predominantly localized in the chloroplasts of salinized *W. biflora* plants, where its concentration may exceed 130 mM (Trossat *et al.*, 1998).

Pinitol concentrations in the chloroplasts isolated from leaves of *M. crystallinum* plants have been estimated to be 230 mM, whereas the cytosol concentration was estimated to be 100 mM [cf. 10 mM on a tissue sap basis] (Paul and Cockburn, 1989). Pinitol was not detected in vacuoles (Paul and Cockburn, 1989). However, as discussed by Popp and Smirnoff (1995) the concentrations of certain cyclitols in the Fabaceae and mangroves can exceed 200 mM on a tissue water basis and it seems highly unlikely

that these could be exclusively localized in the cytoplasm in these species. Vacuolar accumulation of polyols or other carbohydrates may provide osmotic adjustment in saline environments when Na^+ and Cl^- are excluded from leaf tissues (Briens and Larher, 1982). Thus, Briens and Larher (1982) have noted an inverse correlation between leaf inorganic ion content and organic solute concentration among diverse halophytic plant species.

9.5 Concluding discussion

It has long been recognized that salt-tolerant and salt-sensitive plant species differ dramatically with respect to their accumulation of organic solutes, particularly in response to salinity stress (Wyn Jones and Storey, 1981; Blunden and Gordon, 1986; Gorham, 1995; Smirnov and Stewart, 1985; Rhodes and Hanson, 1993; Popp and Smirnov, 1995; Bohnert and Jensen, 1996). Because these organic solutes/osmolytes are non-toxic to enzyme systems across a wide concentration range, and there is sparse but growing evidence that these solutes are accumulated in the cytoplasm, it seems likely that these solutes play a central role in cytoplasmic osmotic adjustment by acting as “compatible cytoplasmic solutes” in plants (Wyn Jones *et al.* 1977), as in other organisms (Hochachka and Somero, 1984; Yancey, 1994). According to Hochachka and Somero (1984) the selection of a particular organic solute for use as an osmotic agent is subsidiary to the fundamental “design principle of establishing a microenvironment for macromolecules in which their structural and functional properties are optimized for catalysis, metabolic regulation, information transfer, and mechanical work, and in which a balance is achieved between stability and instability of structure.” Because many different organic compounds can satisfy this microenvironmental design principle, we should not be surprised by the great diversity of the metabolic pathways of plant osmolyte synthesis discussed above.

Recent studies with transgenic glycophytic plants engineered for the synthesis and accumulation of certain of these compounds (including proline, glycine betaine, mannitol and D-ononitol) indicate that such accumulation may confer modest increases in salt tolerance, and in some cases, increased resistance/tolerance to other stresses (drought, cold, heat or light). In the case of mannitol accumulation it appears that the mode of action of this solute may not be solely in cytoplasmic osmotic adjustment; mannitol may play a more significant role as a hydroxyl radical scavenger in chloroplasts (Shen *et al.*, 1997 b). These advances with model glycophytes, achieved by manipulating single genes, hold substantial promise for designing and implementing engineering strategies to enhance salinity stress resistance of crop plants (Bohnert and Jensen, 1996; Bohnert and Shen, 1999; Jain and Selvaraj, 1997). However, these preliminary genetic interventions have also shown that: (i) the introduction of a foreign gene conferring synthesis of a solute may substantially disturb normal metabolism and growth (Sheveleva *et al.*, 1998; Serrano *et al.*, 1999), and (ii) the engineered gene may often be embedded in a rigid metabolic network that resists change and restricts solute accumulation (Nuccio *et al.*, 1998, 1999). Hare *et al.* (1998) suggest that disturbance of hexose sensing may be an important contributing factor to the stress-tolerance

phenotypes of plants engineered for carbohydrate osmolytes, and that there is a need for re-assessment of the significance of proline and glycine betaine accumulation as mechanisms for buffering cellular redox potential. Further work is needed to identify the unique regulatory features of metabolism that permit the accumulation of substantial levels of compatible solutes found in nature (Jain and Selvaraj, 1997), and to combine these traits with other equally important osmoregulatory features of halophytes discussed in this volume (see Chapter 3), including potassium/sodium discrimination, sodium exclusion, and sodium compartmentation in the vacuole.

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CHAPTER 10

SODIUM-CALCIUM INTERACTIONS UNDER SALINITY STRESS

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Abstract

There are a wide range of responses of plants to salinity which involve interactions of Na with Ca. Plant processes such as growth, photosynthesis, mineral nutrition, water and ion transport are affected by these Na-Ca interactions. Many of these responses can be linked to the direct Na-Ca interactions at the surface of the plasma membrane and subsequent Ca signaling events.

10.1 General Effects of Salinity

Salinity affects plant growth through ionic and osmotic effects. Sometimes these effects are distinct from each other; sometimes these effects overlap. The difference in a plant's response to a given level of salinity is dependent upon the concentration and composition of the ions in solution as well as the genotype that is exposed to the salinity.

Once the importance of Ca in the external solution was fully appreciated (Epstein, 1961), there was a large increase in the number of experiments that focused on the interactions of Na with Ca in plants. Na-Ca interactions are particularly apparent in plants when the Na : Ca ratio in the external solution is above 17 (Greenway and Munns, 1980), but again the plant response will vary with genotype.

10.2 General Characteristics of Na and Ca

10.2.1 PHYSICAL PROPERTIES OF Na AND Ca

10.2.1.1 Na

Sodium (Na), from the Latin word *sodanum*, meaning headache remedy, has a mass of 22.99 and a valence of 1 (Na^+). It is the sixth most abundant element on earth, comprising about 2.6% of the earth's crust. Sodium salts readily dissolve in water. The

crystal ionic radius of Na is 0.097 nm, its hydrated ionic radius has been estimated to be about 0.30 nm, and its heat of hydration is -322 kJ mol^{-1} . The heat of hydration is a measure of how easily a water layer can be stripped from the ion and is important in ion binding, particularly to ion channels.

10.2.1.2 Ca

Calcium (Ca), from the Latin word *calx*, meaning lime, has a mass of 40.08 and a valence of 2 (Ca^{2+}). It is more common than Na, being the fifth most abundant element in the earth's crust (approximately 3%). Ca salts can be quite soluble, but some (i.e. Ca phosphates) are very insoluble. This interaction with phosphate is in part why Ca activities are so low in the cytoplasm (10^{-7} M). The crystal ionic radius of Ca is 0.099 nm, which is very similar to the crystal ionic radius of Na. Because Ca has a valence of 2, Ca^{2+} has a much higher charge density than Na^+ . The higher charge density increases the number of water layers attracted to the ion in solution. Therefore, both its hydrated ionic radius, 0.44 nm, and its heat of hydration, $-1580 \text{ kJ mol}^{-1}$ are much larger than those of Na.

10.2.2 CONCENTRATIONS VS. ACTIVITIES

Ions in saline solutions do not behave ideally. Therefore their activities are usually much lower than their concentrations due to ion pair formation and precipitation (Cramer and Läuchli, 1986). This effect is much more pronounced for Ca than it is for Na. Consequently the Na : Ca ratio in a solution on a concentration basis will be very different from the Na : Ca ratio on an activity basis. Since rates of reaction are dependent upon activities and not concentrations, the interactions of Na with Ca are best considered, when possible, on an activity basis.

10.2.3 NA : CA RATIOS IN SOILS AND WATERS

Based upon the total salt concentration and the Na : (Ca + Mg) ratio, soils have been classified as saline, sodic or saline-sodic (Chapter 2). The total concentration of salts is usually measured by electrical conductivity, EC in units of dS m^{-1} , where 1 dS m^{-1} is approximately equal to a 10 mM concentration of salt that disassociates into two monovalent ions when in solution (e.g. NaCl). Saline soils are commonly defined as those soils having an EC of 4 dS m^{-1} or greater. Sodic soils are defined as those soils that have a sodium adsorption ratio (SAR) greater than 15. SAR is calculated as

$$\text{SAR} = \frac{[\text{Na}^+]}{[\text{Ca}^{2+} + \text{Mg}^{2+}]^{1/2}} \quad (1)$$

where the brackets refer to the concentration in soil solution or the saturated paste extract. In sodic soils, soil colloids disperse, disrupting soil structure and water conductivity. Thus in saline-sodic soils, in which both the EC and SAR are high, this physical change in soil structure has severe consequences on plant growth in addition to the direct effects of the saline solutions. Saline soils and sodic soils make up about 23% and 37%, respectively, of the cultivated land in the world (Chapter 1). Seawater has a

SAR of about 56, whereas lake waters have an average SAR of 0.5 (calculated from Table 2.2 in Epstein, 1972).

10.2.4 ION FUNCTION IN PLANTS

10.2.4.1 Na^+

For most plants, Na is not essential (Marschner, 1995), but the growth of most plants is stimulated at low Na concentrations. Some halophytes require Na for growth (Chapters 3, 16), particularly C_4 and CAM plants, and some halophytes require very high concentrations of Na for maximal growth (Flowers *et al.*, 1977). Na is generally used as an osmoticum in the vacuole, usually reducing the plant need for K (Marschner, 1995), whereas some halophytes have specific enzymatic needs for Na as well (Cramer, 1997).

10.2.4.2 Ca^{2+}

Ca is an essential element in all plants (Marschner, 1995). The ability of Ca to form intermolecular linkages gives it an important role in maintaining the integrity and structure of membranes and cell walls (Hanson, 1984). Ca is also used as a second messenger in many signal transduction pathways within the cell (Bush, 1995).

10.3 Na-Ca Interactions

10.3.1 EFFECTS ON PLANT GROWTH

The ameliorative effects of Ca on Na toxicity in plants has been reported as far back as 1902 (see references in LaHaye and Epstein, 1971). There were only a few papers that addressed this topic in the first half of the 20th century (see references in LaHaye and Epstein, 1971). Na-Ca interactions were largely overlooked until the importance of external Ca on the ion selectivity of ion transport was realized (Epstein, 1961). Since this discovery, there have been a very large number of papers published on Na-Ca interactions in plants, most notably in the last two decades. Because of the large number of papers, it is only possible to cite a few examples to illustrate the most important points. The reader is referred to several excellent reviews on Na-Ca interactions in plants for further information (Greenway and Munns, 1980; Läuchli and Schubert, 1989; Rengel, 1992; Lazof and Bernstein, 1999).

Although Ca ameliorates the Na-inhibition of growth for most plants, this is not always the case. During my graduate studies at the University of California, Davis, I tested the response to salinity and supplemental Ca of every species we were using in the lab at that time. Different species and different cultivars responded differently to supplemental Ca when salinized (Table 1).

Most genotypes responded favorably to supplemental Ca when salinized. A couple of genotypes actually responded negatively (Kenaf and *Thinopyrum ponticum*). Kenaf showed interveinal chlorosis in the lower leaves, similar to Mg deficiency symptoms.

TABLE 1. Growth response of different species to salinity with high (10 mM) or low (0.4 mM) Ca^{2+} in the nutrient solution (0.1 modified Hoagland, pH 5.5). Eleven-day-old plants were salinized with 25 mM NaCl day⁻¹ increments until the final concentration of 100 mM NaCl was reached. Plants were harvested one week after the final concentration was reached. Data are means of 8 plants for each treatment. A rank of number 1 means the highest response to supplemental Ca (G.R. Cramer, unpublished results).

Plant/ Genotype	Fresh Weight (g)				High Ca/Low Ca (%)		Rank for Positive Response to Ca	
	0.4 mM Ca^{2+}		10 mM Ca^{2+}		Root	Shoot	Root	Shoot
	Root	Shoot	Root	Shoot				
Barley								
Arivat	2.51	2.80	3.66	3.62	146	129	11	10
Calmariot	1.32	1.17	2.04	1.77	155	151	10	5
Triticale								
Siskiyou	0.99	0.86	2.13	1.68	215	195	4	2
GTA 208	0.98	0.83	1.83	1.21	187	146	6	7
Wheatgrass								
T. intermedium	0.05	0.06	0.12	0.12	240	200	1	1
T. ponticum	0.17	0.22	0.16	0.16	94	73	14	14
Maize								
Pioneer 3906	2.7	5.68	6.44	10.09	239	178	2	3
Dekalb XL75	1.40	4.86	3.14	6.75	224	139	3	8
Pioneer 3377	3.51	8.67	5.91	11.70	168	135	8	9
Tomato								
VF36	0.18	0.62	0.36	1.08	200	174	5	4
Edkawi	0.25	0.77	0.41	1.16	164	150	9	6
Sorghum								
S. bicolor	1.60	1.34	1.70	1.60	106	119	13	11
Kenaf								
C-108	1.62	2.87	1.31	1.87	81	65	15	15
Cotton								
Acala S-J2	2.20	5.23	2.52	5.2	115	99	12	13
Bean								
Phaseolus vulgaris	0.75	1.75	1.30	2.06	173	118	7	12

In addition, there were noticeable differences between cultivars within a species, especially within maize.

Other scientists have also found different genotype responses to supplemental Ca and salinity within rice (Yeo and Flowers, 1985; Grieve and Fujiyama, 1987; Muhammed *et al.*, 1987), *Brassica* species (Ashraf and Naqvi, 1992; Schmidt *et al.*, 1993), *Hordeum* species (Suhayda *et al.*, 1992), maize (Maas and Grieve, 1987; Alberico and Cramer, 1993), blueberry (Wright *et al.*, 1992), sorghum (Grieve and Maas, 1988) and citrus (Zekri, 1993). It is hoped that the reader will keep in mind the differential responses of plants to Ca, especially when broad generalizations are made in this review and elsewhere.

The effect of supplemental Ca on salt-stressed roots has been related to the ion activities in the external solution (Cramer *et al.*, 1986; Yermiyahu *et al.*, 1997). Originally, root elongation was correlated to the Na : Ca activities in solution (Cramer *et al.*, 1986) based upon a simple ion exchange theory at the surface of the plasma membrane (Cramer and Läuchli, 1986). Recently, a much more sophisticated approach has been fully developed which includes osmotic effects along with ionic effects (Yermiyahu *et*

al., 1997; Kinraide, 1999). The latter approach appears to be widely applicable to many different ionic conditions.

The level of Ca in the external solution needed for maximal growth in saline conditions is usually between 5 and 10 mM Ca depending on the salinity level and genotype (see Table 2); concentrations of Ca above 10 mM can inhibit plant growth. The optimal Na : Ca ratio is somewhere between 10 and 20 for most plants tested (Table 2). To the best of my knowledge no such studies have been carried out in detail for halophytes.

TABLE 2. Ca concentrations necessary for maximal growth of salinized plants.

Species	Na (mM)	Ca (mM)	Na : Ca	Reference
<i>Phaseolus vulgaris</i>	80	5	16	(Cachorro <i>et al.</i> , 1993 a)
	50	3	17	(LaHaye and Epstein, 1971)
	24	1	24	(Wadleigh and Bower, 1950)
<i>Zea mays</i>	71	12.5	6	(Maas and Grieve, 1987)
<i>Vaccinium ashei</i>	100	1	100	(Wright <i>et al.</i> , 1992)
<i>Cucumis melo</i>	40-100	5	8-20	(Yermiyahu <i>et al.</i> , 1997)
<i>Triticum aestivum</i>	50	5	10	(Hawkins and Lewis, 1993 b)
<i>Vigna mungo</i>	60	3.5	17	(Nakamura <i>et al.</i> , 1990)
	80	6	13	
	100	5	20	
	150	7	21	
<i>Sorghum bicolor</i>	86	2.5	34	(Grieve and Maas, 1988)
	71	12.5	6	
	71	12.5	6	
	108	17.8	6	
	64.8	43.1	4	
	108	17.8	6	
<i>Oryza sativa</i>	86	4	18	(Grieve and Fujiyama, 1987)
	95	4.8	20	(Muhammed <i>et al.</i> , 1987)
Average		8.9	19	

The effects of supplemental Ca on the growth of salt-stressed roots are immediate (Cramer *et al.*, 1988; Zhong and Läuchli, 1993 b). If 80 mM NaCl was added before addition of supplemental Ca, then root growth was strongly inhibited and only slowly recovered over several hours (Cramer *et al.*, 1988). However, if the Ca concentration in the nutrient solution was high, then root growth was unaffected by addition of 80 mM NaCl to the nutrient solution (Cramer *et al.*, 1988). In addition, differences in leaf growth of maize (Cramer, 1992) and bean (Ortiz *et al.*, 1994) are discernable within hours after varying Na : Ca salinities.

Cell expansion is a function of water uptake and cell wall extension. It involves both biochemical and physical processes (Figure 1). The current view is that a biochemical loosening of the cell wall under turgor pressure allows cell expansion to proceed followed by the nearly simultaneous absorption of water and solutes (Hsiao *et al.*, 1976; Boyer, 1987; Cosgrove, 1987).

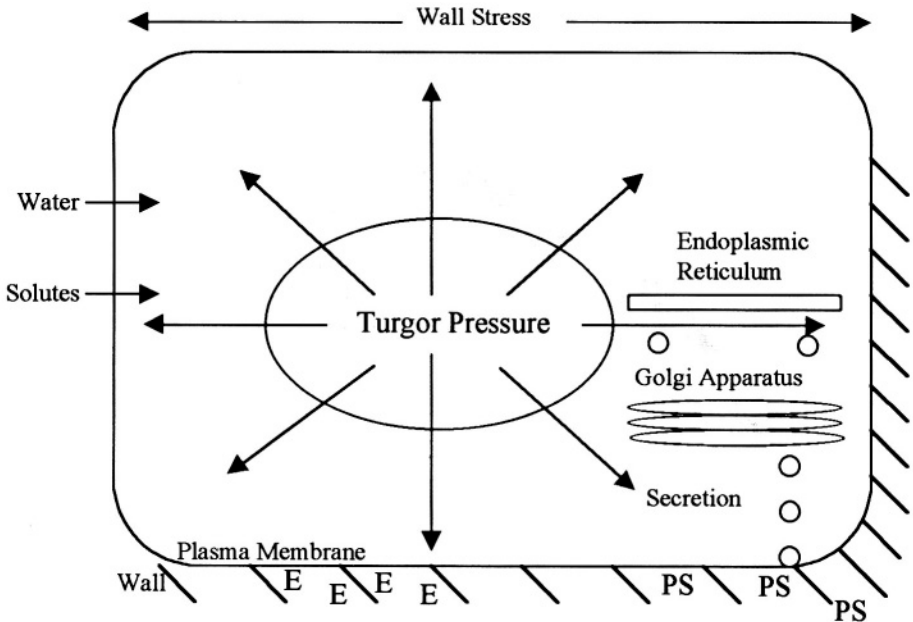


Figure 1. The expansion of a plant cell is dependent upon a physical stress that stretches the cell wall (turgor) and biosynthetic processes involving cell wall enzymes (E) and polysaccharides (PS).

A rigorous description of growth includes both mechanical and hydraulic aspects (Boyer, 1987):

$$\frac{1}{V} \frac{dV}{dt} = \frac{mL}{m+L} (\psi_o - \psi_s - Y) \quad (2)$$

This is a variation of a previously derived equation (Lockhart, 1965), where m , L , ψ_o , ψ_s , and Y represent the cell wall extensibility, the hydraulic conductance, the xylem water potential, the cell osmotic potential, and the yield threshold, respectively. V is the cell volume and t is time. The term, $mL/(m+L)$, is often referred to as the growth coefficient. The term, $(\psi_o - \psi_s - Y)$, is the driving force for cell expansion. The yield threshold is the minimum turgor at which cells expand. Equation 2 is very useful for the analysis of growth limitations.

The way in which salinity and supplemental Ca affect growth has been analyzed for maize leaves (Cramer, 1992) using this mechanistic approach. Once steady-state conditions were reached after application of salinity, leaf growth was inhibited solely by an increase in the yield threshold of the cell wall (Cramer and Bowman, 1991). After a day of salinization, the cell wall extensibility was decreased as well (Cramer, 1992). In plants grown with a high Na : Ca ratio, the hydraulic conductance was reduced; supplemental Ca (10 mM) improved growth by restoring hydraulic conductance back to that of the control plants (Cramer, 1992).

Supplemental Ca can affect the length of the growth zones of salt-stressed plants. In sorghum leaves, the length of the growth zone is shortened by 100 mM NaCl salinity. If the Ca concentration of the nutrient solution is increased from 1 to 10 mM, then shortening of the growth zone by salinity is prevented (Bernstein *et al.*, 1993). However, supplemental Ca did not influence the length of the growing zone in salt-stressed cotton roots (Zhong and Läuchli, 1993 b).

10.3.2 EFFECTS ON CELL SHAPE, SIZE AND PRODUCTION

Figure 1 is a simplistic model for unidirectional (longitudinal) cell expansion. However, cells expand in a radial direction as well. The direction of expansion has pronounced effects on cell shape. The external Na : Ca ratio in solution can have significant effects on cell shape and cell production. In cotton roots, a high Na : Ca salinity causes cortical cells to become nearly isodiametric in contrast to controls (Kurth *et al.*, 1986). Root cells exposed to low Na : Ca salinity become much longer and thinner. In both cases, however, there was no change in total volume of the cells. Supplemental Ca stimulated cell production in cotton roots by about 20%. In roots treated with 0.4 mM Ca, cell production was inhibited at 50 mM NaCl. In roots treated with 10 mM Ca, cell production was inhibited at 200 mM NaCl.

In maize roots, 100 mM NaCl reduced cell length in the root cortex by about half compared to controls (Zidan *et al.*, 1990; Azaizeh *et al.*, 1992). Plant roots grown with supplemental Ca (10 mM final concentration) without salinity had slightly reduced cell lengths compared to controls in one study (Azaizeh *et al.*, 1992) and were not different from controls in another (Zidan *et al.*, 1990). Plant roots grown with salinity and supplemental Ca had root cell lengths restored to slightly less than that of controls (Zidan *et al.*, 1990; Azaizeh *et al.*, 1992) and equivalent to the 10 mM Ca-grown roots in one study (Zidan *et al.*, 1990). Cell volume was also reduced in salinity treatments without supplemental Ca, but not in treatments with supplemental Ca (Azaizeh *et al.*, 1992). Root growth and cell production rates of salt-stressed maize were partially restored with supplemental Ca (Zidan *et al.*, 1990). Possible mechanisms involved in the effects of Ca on cell expansion are discussed in Section 3.5.

10.3.3 EFFECTS ON PHOTOSYNTHESIS

Na-Ca interactions on photosynthesis have been observed (see also Chapter 15). In *Citrus sinensis*, growth is very sensitive to Cl^- concentrations (Bañuls and Primo-Millo, 1992). NaCl and KCl salinity treatments significantly reduce photosynthesis, but isosmotic concentrations of NaNO_3 had no significant effects. When Ca concentrations were increased to 30 mM with Ca acetate, photosynthetic rates were similar to that of controls. The higher rates of photosynthesis were attributed to lower concentrations of Cl^- in the leaves. In *Vaccinium ashei*, another species extremely sensitive to Cl^- , supplemental Ca increased photosynthetic rates in salt-stressed plants treated with Na_2SO_4 , but not with NaCl (Wright *et al.*, 1993). In both species, growth was highly correlated with photosynthesis. Since these were long-term experiments, it is unclear which was the cause and which was the effect; photosynthesis can affect the growth rate of plants, but it can also be feedback-regulated by growth rates.

There are specific Na-Ca interactions on stomatal conductance. In abaxial epidermal peels of *Aster tripolium*, a halophyte, stomatal conductance is inhibited by external Na concentrations, whereas in *Commelina communis*, a nonhalophyte, external Na concentrations stimulate stomatal conductance (Perera *et al.*, 1994). When Ca is included in the medium in the presence of 50 mM NaCl, Ca concentration prevents the reduction of stomatal conductance in *Aster tripolium* (Perera *et al.*, 1995). However, in the presence of 50 mM KCl, Ca concentrations reduce stomatal conductance (Perera *et al.*, 1995). These responses are likely to be due to Na-Ca interactions at the plasma membrane of the guard cells and their effects on Na, K, and Ca fluxes (Perera *et al.*, 1995).

In other plant species, no Na-Ca interactions have been observed on stomatal conductance and photosynthesis. In sunflower, there were differential responses between two lines. Transpiration and stomatal conductance were unaffected by varying Na : Ca ratios (all salt solutions were at 150 mM NaCl) in one line, whereas in another line there were significant reductions as the Na : Ca ratio was increased (Ashraf and O'Leary, 1997). Although the effect of varying Na : Ca ratios on photosynthesis was not statistically significant in sunflower, there was a slight trend towards reduced photosynthesis with increasing Na : Ca ratios (Ashraf and O'Leary, 1997).

Salinity reduced transpiration in barley, but supplemental Ca did not alter transpiration rates (Cramer *et al.*, 1989). In wheat (Hawkins and Lewis, 1993 a) and cotton (Leidi *et al.*, 1991), no significant effects of supplemental Ca were observed in salt-stressed plants on stomatal conductance, transpiration, and photosynthesis. In salt-stressed maize, varying Na : Ca ratios did not have significant effects on transpiration and photosynthesis (Plaut and Grieve, 1988) or the net assimilation rate (Cramer *et al.*, 1994 a). Thus, it would appear that in some species Na : Ca interactions affect growth without affecting photosynthesis.

10.3.4 EFFECTS ON WATER TRANSPORT

Water transport is a potentially limiting factor in plant growth (Boyer, 1985; Passioura, 1988; Steudle, 1989). Water transport is usually measured as the hydraulic conductivity (L_p), which is a measure of water flow across an individual unit area of membrane. L_p does not take into account the complex pathways of water transport through many cells and whole tissues. Hydraulic conductance (L) does measure the average water conductance of a pathway and is related to L_p ($L = L_p \times A$ where A is the average area of the pathway).

Water transport into the root and to the leaf growing zone is affected by Na : Ca ratios. As mentioned above, salinity reduces hydraulic conductance (L) to the leaves of maize plants treated with NaCl salinity for 24 h (Cramer, 1992), but has no effect on plants treated for 4 h (Cramer and Bowman, 1991). Supplemental Ca fully prevents the inhibition of hydraulic conductance, but leaf elongation rates remain partially inhibited (Cramer, 1992).

The hydraulic conductivity of roots (L_{pr}), which is the root hydraulic conductance divided by the effective root surface area, includes both apoplastic and symplastic water transport pathways of the root (Azaizah *et al.*, 1992). Both L_{pr} (Evlagon *et al.*, 1990;

Azaizeh and Steudle, 1991) and the Lp (Azaizeh *et al.*, 1992) of root cells of maize are reduced by NaCl salinity. The inhibition of Lpr was partially (Evlagon *et al.*, 1990) or fully (Azaizeh and Steudle, 1991) prevented by supplemental Ca. The inhibition of root cell Lp was also prevented by supplemental Ca (Azaizeh *et al.*, 1992). Lp is inhibited much more than Lpr by salinity indicating that salinity and supplemental Ca primarily affect water transport across cell membranes rather than the apoplastic transport pathway (Azaizeh *et al.*, 1992). The reduction of water transport across the plasma membrane may be due to salinity effects on cytosolic Ca and the dephosphorylation of water channels (see Sec. 3.7.).

10.3.5 EFFECTS ON CELL WALL

Nearly 50% of cellular Ca is bound in the cell wall to carboxyl groups, particularly in pectins (Demarty *et al.*, 1984; Hanson, 1984). Ion exchange theory for cell walls is well developed (Grignon and Sentenac, 1991) and there are clear Na-Ca interactions in the cell wall (Demarty *et al.*, 1984; Zid and Grignon, 1985). Under nonsaline conditions, Na : Ca ratios are correlated to differentiation in secondary walls (Ripoll *et al.*, 1993). In salinized *Citrus aurantium*, Na competes with Ca for anionic sites in the leaf cell wall which have a high specificity for Ca (Zid and Grignon, 1985). In addition to the interference with cell wall Ca function, the localization of high concentrations of Na in the cell wall may lead to plant injury by reducing cell turgor (Oertli, 1968; Flowers *et al.*, 1991).

In maize leaves, the short-term, steady-state limitations of cell expansion by salinity are associated with cell wall hardening (an increase in what appears to be the cell wall yield threshold) and not to hydraulic or turgor effects (Cramer and Bowman, 1991; Cramer and Schmidt, 1995; Neumann, 1995). Prior to steady-state conditions, turgor is rapidly reduced, but recovers to that in controls in a matter of hours (Thiel *et al.*, 1988; Cramer and Bowman, 1991). Roots respond differently than leaves in that they are much less sensitive to salinity (Munns and Sharp, 1993). This can be attributed to an increase in cell wall loosening (Frensch and Hsiao, 1995; Wu *et al.*, 1996 b), the opposite response of leaves.

There are few data on the effect of Na : Ca ratios on cell wall extension properties of salinized plants (Lynch *et al.*, 1988; Cramer, 1992). These data indicate that both the cell wall extensibility and yield threshold of the cell wall were not affected by varying Na : Ca ratios in a manner that would contribute to the reduction of growth and size of salt-stressed leaves. There are no reports on the effects of Na : Ca ratios on root cell wall extension properties. Thus, there is insufficient evidence to make any generalizations on this subject.

In barley, Na : Ca ratios in expanding leaf tissue increased with increasing salinity and leaf growth was reduced significantly (Lynch *et al.*, 1988). Salinity did not decrease the *in vitro* plastic compliance of these tissues; a decrease in plastic compliance would be predicted by the high Na : Ca ratio in the tissue (Lynch *et al.*, 1988). Similar observations have been made for *in vitro* assays in maize (G.R. Cramer, unpublished results). If anything, plastic compliance was increased by salinity, even though salinity significantly reduced leaf growth (Lynch *et al.*, 1988). If cell walls are hardened in these

plants, then it must be by some other mechanism, perhaps by a reduced secretion (Cramer and Jones, 1996) or reduced biosynthesis of cell wall components *in vivo*.

Na : Ca ratios do affect cell wall biosynthesis (Zhong and Läuchli, 1988; Zhong and Läuchli, 1993 a). Salinity at a high Na : Ca ratio inhibits cellulose and noncellulosic polysaccharide biosynthesis of the cell walls of cotton roots (Zhong and Läuchli, 1988). At a low Na : Ca ratio, only noncellulosic polysaccharide biosynthesis of the cell wall is inhibited. High Na : Ca ratios increased the uronic acid content and decreased the cellulose content of the cell wall (Zhong and Läuchli, 1993 a). Supplemental Ca prevented these changes in uronic acid and cellulose content. The neutral sugar content of the cell wall was unaffected by Na : Ca ratios, but there was a shift in polysaccharide molecular size (Zhong and Läuchli, 1993 a). It was suggested that polysaccharide degradation and enzymatic activities in the cell wall might be inhibited by high Na : Ca ratios. However, cell wall enzymes involved in cell expansion from both halophytes and nonhalophytes are relatively salt tolerant compared to cytoplasmic enzymes and are unlikely to be the cause of reduced growth by salinity (Thiyagarajah *et al.*, 1996). It has been suggested that changes in cell wall composition by salinity, particularly that of pectic polysaccharides, may be the result of a salinity-induced Ca deficiency (Kafkafi and Bernstein, 1996).

10.3.6 EFFECTS ON MEMBRANES

From the early observations of Na : Ca interactions on plant growth (see Sec. 3.1. above) and the development of the Gouy-Chapman theory for membranes, it was logical to hypothesize that Na : Ca interactions occurs at the surface of membranes (see LaHaye and Epstein, 1969 and references therein). Since membranes form compartments and are sites of many biological functions, interference of Na with Ca function in membranes of salt-stressed plants could have very serious consequences, particularly involving ion transport and compartmentation.

The first direct evidence for Na : Ca interactions at the membrane in salt-stressed plants was provided by the use of chlortetracycline (CTC) as a fluorescent probe for membrane-associated Ca (Cramer *et al.*, 1985). In salt-stressed roots of cotton, Na displaced membrane-associated Ca, which was believed to be primarily located at the plasma membrane (Cramer *et al.*, 1985). In other experiments where membrane-associated Ca was measured directly, NaCl-salinity also displaced membrane-associated Ca on protoplasts of corn (Lynch *et al.*, 1987; Lynch and Läuchli, 1988) and barley (Bittisnich *et al.*, 1989), and on plasma membrane vesicles of melon (Yermiyahu *et al.*, 1994).

In corn root protoplasts, evidence was provided that part of the Ca-CTC fluorescence may come from internal membrane-bound compartments within the cell which may contain high Ca concentrations (Lynch *et al.*, 1987; Lynch and Läuchli, 1988). Li blocks the release of Ca from internal stores caused by phosphoinositides. When cells were pretreated with Li, membrane-associated Ca was not reduced by 150 mM NaCl as much as in cells without Li pretreatment. Cells pretreated with Li and inositol (which is needed for the regeneration of phosphoinositides) behaved like cells without pretreatment; in other words, membrane-associated Ca was reduced to the same extent by salinity. Cytoplasmic Ca responded in a similar manner to Li, inositol and salinity

(Lynch and Läuchli, 1988), consistent with the conclusion that salinity can release Ca from internal compartments that store Ca (Lynch *et al.*, 1987).

Displacement of membrane-associated Ca is different at different Ca concentrations in the solution (Cramer *et al.*, 1985; Lynch *et al.*, 1987); there is a greater displacement of membrane-associated Ca by Na at low external Ca concentrations. In addition, salt-tolerant genotypes of barley (Bittisnich *et al.*, 1989) and melon (Yermiyahu *et al.*, 1997) had less displacement of membrane-associated Ca by NaCl-salinity than salt-sensitive genotypes of the same species. These data indicate that the partial alleviation of NaCl inhibition of growth by Ca may in part be related to the quantity of membrane-associated Ca.

Consistent with this hypothesis, the salt tolerance of four melon varieties was associated with the greater ability of their root membranes to bind Ca (Kafkafi, 1991). The ability to bind Ca was cleverly deduced from root responses to monovalent salts and comparison to ion selectivity at a surface according to Eisenman's series. Ion selectivity is a function of the relative energy of interaction between the ion's hydration energy and the surface charge density of the adsorbing surface (Kafkafi, 1991).

NaCl-salinity also reduces the surface potential of the plasma membrane (Suhayda *et al.*, 1990), which is a function of the surface charge density and the ion activity in the surrounding solution. Salt reduced the surface potential directly by screening negative charges on the membrane and also indirectly in plants that had been salt-stressed for four days (presumably by reducing the surface charge density of the membrane over time). A reduction in charge density would reduce the cation activity at the outside surface of the plasma membrane and therefore affect rates of ion transport across the plasma membrane.

Sophisticated models have been developed and applied to analyze Na : Ca interactions and estimate Ca-binding at the plasma membrane surface of plants (Kinraide, 1994; Yermiyahu *et al.*, 1997; Murata *et al.*, 1998 a). These models use measurements of surface potential to estimate the surface charge density. They also calculate ion activities at the surface of the membrane based upon known ion chemistry in solution. From these parameters, the amount of Ca binding to the membrane can be computed. Using this indirect approach, these models estimate that Na reduces membrane-associated Ca (Kinraide, 1994; Yermiyahu *et al.*, 1997; Murata *et al.*, 1998 a).

It was discerned from ion exchange analysis that Ca was displaced from two different classes of sites, one being a high-affinity binding site, probably associated with proteins, and the other being a low-affinity site, probably associated with phospholipids (Cramer and Läuchli, 1986). Other reports also provide evidence for multiple Ca-binding sites at the plasma membrane of other plant species (Yermiyahu *et al.*, 1994; Murata *et al.*, 1998 a). Displacement of Ca from different sites will cause different effects.

In intact cotton roots, displacement of membrane-associated Ca from root hairs appears to be specific for Na; treatments with other monovalent cations, Ba (a divalent cation), or mannitol do not reduce membrane-associated Ca. Therefore, it is assumed that this effect is Na-specific. However, in corn root protoplasts isolated from the cortex, in addition to the displacement of membrane-associated Ca by Na, other monovalent cations displace membrane-associated Ca (Lynch *et al.*, 1987). In this study, all

monovalent cations substantially reduced membrane-associated Ca. Very small but significant differences were found between cations; the effect was in the following order: $\text{Li} = \text{K} = \text{Rb} > \text{Na} > \text{Cs}$. The lack of substantial difference between cations indicates that displacement of Ca was caused by an ionic strength effect, which was nonspecific. Likewise, it should be noted that all protoplasts were in isosmotic solutions, therefore these effects were truly ionic without any osmotic component. Based upon unpublished work on intact corn roots the authors suggested that the differential responses of ion specificity between corn and cotton were genetically based and not due to the differences in methods (i.e. intact cells vs. protoplasts). In support of this conclusion, Na-specific effects were recently identified in tobacco, but nonspecific ionic effects were identified in barley (Murata *et al.*, 1998 a). Thus, different genotypes seem to have inherently different responses.

One consequence of the displacement of membrane-associated Ca by Na is the immediate increase of K efflux across the plasma membrane of salt-stressed cotton roots at salt concentrations above 100 mM NaCl (Cramer *et al.*, 1985). Supplemental Ca reduces this effect at concentrations above 150 mM NaCl. Isosmotic concentrations of mannitol have similar effects as saline treatments with supplemental Ca (10 mM) indicating that K efflux is affected by osmotic factors in these solutions and not associated with a Na-specific displacement of membrane-associated Ca (Cramer *et al.*, 1985). This effect may be related to the rapid depolarization of the membrane potential upon salinization (Cramer, 1997).

Salinity and supplemental Ca can alter lipid composition of plant membranes (Cachorro *et al.*, 1993 b; Yu *et al.*, 1998). In beans and barley, salinity reduced total phospholipid content of the membrane (Cachorro *et al.*, 1993b; Yu *et al.*, 1998). This might account for the reduction in surface charge density described above. Furthermore, supplemental Ca increased the phospholipid content in both cases. Salinity and Ca also affected the fatty acid compositions. Specifically, the content of unsaturated fatty acids increased with salinity and was reduced by supplemental Ca (Cachorro *et al.*, 1993 b; Yu *et al.*, 1998). An increase in the proportion of unsaturated fatty acids causes an increase in K permeability across membranes (Scarpa and de Grier, 1971). This effect is not likely to be related to the K efflux cited in the previous paragraph, since lipid composition would be unaffected in those short-term experiments.

10.3.7 EFFECTS ON ION TRANSPORT AND CONTENT

There is abundant evidence that salinity alters the ion transport and contents of plants (Cramer, 1997). In general, Na uptake and concentrations increase and Ca uptake and concentrations decrease in plant cells and tissues as the external Na concentration increases (Rengel, 1992; Cramer, 1997; Lazof and Bernstein, 1999). Likewise, as external Ca concentrations increase Na uptake and concentration decrease and Ca uptake and concentration increase. One consequence of these Na : Ca interactions is the reduction of K content in salinized plants, which can be prevented with supplemental Ca.

The issue of Na toxicity in plants has been addressed extensively in previous reviews and will not be addressed here (see Flowers *et al.*, 1977 and references therein; Greenway and Munns, 1980; Cramer, 1997; Lazof and Bernstein, 1999). However, it

should be noted that just because Na and Cl tissue concentrations increase with salinity (which is a function of increasing external concentrations and uptake) it does not necessarily mean that these concentrations are the cause of the growth reduction. In many cases, especially at low to medium salinity stresses, Na toxicity is probably not the cause of growth reduction (Cheeseman, 1988; Munns *et al.*, 1988; Lazof and Lauchli, 1991; Cramer *et al.*, 1994b; Bernstein *et al.*, 1995; Leidi and Saiz, 1997).

The issue of Ca deficiency is not as extensively studied as Na toxicity. Ca deficiency can occur in some species at high Na : Ca ratios (Maas and Grieve, 1987; Muhammed *et al.*, 1987; Ehret *et al.*, 1990; Francois *et al.*, 1991; Ho and Adams, 1994; Bernstein *et al.*, 1995; Fortmeier and Schubert, 1995). Bulk tissue Ca concentrations and Ca influx are reduced by salinity (Lynch and Lauchli, 1985; Cramer *et al.*, 1987; Lynch and Lauchli, 1988; Cramer *et al.*, 1989; Cramer *et al.*, 1994b; Davenport *et al.*, 1997; Halperin *et al.*, 1997; Lazof and Bernstein, 1999) and these factors certainly contribute to Ca deficiency.

Reduction of Ca by salinity has also been detected in the apical meristem and young leaves of lettuce by electron-probe microanalysis (Lazof and Lauchli, 1991) and all along the elongation zone of sorghum leaves (Bernstein *et al.*, 1995). Ca content of growing tissue under salinization can be reduced, and is restored in the same region under elevated Ca levels (Lazof and Bernstein, 1999). This reversal of the reduction in Ca level corresponds with partial reversal of the growth inhibition and prevention of the reduction in the growing zone length (Lazof and Bernstein, 1999).

In some cases, Ca imbalance may not be detectable by normal methods. Salinity may disturb normal Ca functions without disturbing overall Ca tissue concentrations. It can do this because cytoplasmic Ca activities are in the nM range, whereas overall tissue concentrations are in the mM range. It is very difficult to measure changes in Ca activity by conventional means (e.g. atomic absorption spectrophotometry); instead sophisticated fluorescent and luminescent techniques must be employed (Gilroy *et al.*, 1989; Bush and Jones, 1990; Knight *et al.*, 1997).

The effect of salinity on cytoplasmic Ca has been studied using these techniques. There are a variety of responses. Initial studies using maize root protoplasts indicated that salinity increased cytoplasmic Ca activities at high salinity (Bittisnich *et al.*, 1989; Lynch *et al.*, 1989). Recently, cytoplasmic Ca activities in whole seedlings of *Arabidopsis* were found to increase (at least transiently) in response to high salinity or mannitol (Knight *et al.*, 1997). Cytoplasmic Ca activity peaked within 5 to 10 s after exposure to osmotic stress, declining thereafter. Measurements returned close to control values after 60 s. It is not known what happens after 60 s because measurements were terminated by 60 s. The characteristics of these transient responses seem to vary with environmental stimuli (Malho *et al.*, 1998) and may be responsible for different physiological responses to these stimuli. Furthermore, it appears that the previous history or exposure to other stimuli can affect the response of cytosolic Ca to a current stimulus (Knight *et al.*, 1998).

In tissue culture cells of two *Brassica* species, salinity caused a multitude of responses. In some cells, cytoplasmic Ca was increased, in other cells there was no effect, and in other cells, cytoplasmic Ca was decreased (He, 1993). Salinity decreased cytoplasmic Ca activities in the roots of maize (Cramer, 1997) and *Arabidopsis* (Cramer and Jones,

1996; Cramer, 1997). In addition, mannitol treatments decreased cytoplasmic Ca activity in *Arabidopsis* roots (Cramer and Jones, 1996) and tobacco cells (Jones *et al.*, 1998). One salinity treatment caused a transient increase in cytoplasmic Ca activity in *Arabidopsis* roots 6 s after exposure to salinity, but decreased below control values by 69 s (Cramer, 1997). It should also be noted that a sudden decrease in osmotic stress (hypoosmotic) causes a sudden transient increase in cytoplasmic Ca (Taylor *et al.*, 1996; Takahashi *et al.*, 1997; Taylor *et al.*, 1997).

All of the above treatments were applied as rapid osmotic perturbations of the cells over a period of 15 minutes or less. These results indicate that this transient response of cytoplasmic Ca activity to salinity is variable depending upon the cell type, salinity concentration and length of exposure. To my knowledge, there are no long-term, steady-state studies on the effects of salinity on cytoplasmic Ca activities. With the inhibition of Ca transport into plant cells by salinity, it is difficult to see how cytoplasmic Ca activities can remain elevated over a substantial period of time.

What controls the changes in cytoplasmic Ca activity during salt stress and which adjacent Ca pools are affected? This is unclear. In intact plant cells tested so far, the response is osmotic, and not specifically related to Na antagonism (Cramer and Jones, 1996; Knight *et al.*, 1997). In corn root protoplasts, which by necessity must be kept in isosmotic solutions, cytoplasmic Ca increased upon a substantial increase in ionic strength of the external medium (Lynch and Läuchli, 1988; Bittisnich *et al.*, 1989). An increase in external Ca will also cause a rise in cytoplasmic Ca activity, suggesting that Ca influx across the plasma membrane contributes to the increase in cytoplasmic Ca activity. However, this rise in Ca is blocked if cells are already salinized (Cramer and Jones, 1996). Some Ca can be released from internal stores (Lynch and Läuchli, 1988). It is not known how salinity affects cytoplasmic Ca when external Ca concentrations are high. Further research is needed to understand Ca dynamics and compartmentation in salt-stressed plants over longer periods of time when growth rates are in steady-state conditions.

Great progress has been made in characterizing Na and Ca transport across membranes. It is now clear that Na can enter cells through ion channels (for a more extensive review see Amtmann and Sanders, 1998; Tyerman and Skerrett, 1999). In some cases these channels are more selective for Na than K (Roberts and Tester, 1997 a). Increasing the external Ca reduces Na conductance through these channels (Roberts and Tester, 1997a; Tyerman *et al.*, 1997) and this effect is highly correlated with effects on Na influx into roots (Tyerman and Skerrett, 1999). Na influx into membrane vesicles (Allen *et al.*, 1995) and roots (Davenport *et al.*, 1997) is more sensitive to Ca in a salt tolerant species of wheat than a salt-sensitive species. Multiple mechanisms for Na entry exist within plants with at least one mechanism insensitive to Ca (Amtmann and Sanders, 1998; Tyerman and Skerrett, 1999).

Ca entry into cells can also occur through ion channels (Muir *et al.*, 1997; Piñeros and Tester, 1997; White, 1998 b; White, 1998 a). These channels are also permeable to Na (White, 1998 a), but it is not certain how Na interacts with Ca in these channels. K also moves through a Ca channel and can interfere with Ca transport (Piñeros and Tester, 1997); it seems likely that Na would do the same.

An outward rectifying cation channel has also been discovered in maize stelar cells (Roberts and Tester, 1997 b). These channels may control the transport of cations to the xylem (and therefore may control cation transport to the shoot). These channels are preferentially selective for K, but Na can also move through them to a lesser extent. Movement of Na through these channels would likely reduce K movement through them. These channels also appear to be permeable to Ca although Ca entry is predicted to be from the opposite side (apoplast). These ion interactions with the outward rectifying cation channel are consistent with the observed transport of these ions from the root to the shoot in salt-stressed maize (Cramer *et al.*, 1994 b). It is interesting to note that channels in root cortical cells have very different properties (Roberts and Tester, 1997 b).

Calcium did not affect the K/Na selectivity of K outward rectifying cation channels in two wheat genotypes differing in salt tolerance (Schachtman *et al.*, 1991) or in tobacco cells (Murata *et al.*, 1998 b). It is not known what type of cells these channels came from and therefore they cannot be properly compared to the maize channels described in the paragraph above.

In addition to the direct effects of Ca on ion transport, Ca may act on transport through a Ca signaling pathway. Recently, a genetic approach has been applied to salt-stressed *Arabidopsis* mutants (Wu *et al.*, 1996a; Zhu *et al.*, 1998). These mutants are hypersensitive to salt and defective in their K nutrition. One of these mutants, *sos3* (salt-overly-sensitive 3), requires increased Ca for its K nutrition and salt tolerance (Liu and Zhu, 1997). Under salinity stress, this mutant acquires more Na and less K. In addition, this mutant is unable to grow with low external K concentrations. With supplemental Ca the *sos3* mutant grows normally and has improved salt tolerance. *SOS3* encodes a protein that appears to be very similar to a subunit of Calcineurin B in yeast and neuronal calcium sensors in animals (Liu and Zhu, 1998). Because of these similarities it is believed that this protein is involved in a Ca signaling pathway which regulates Na and K transport and thus can alter the K/Na selectivity of the plant (Liu and Zhu, 1998).

Likewise, expression of a yeast calcineurin in transgenic tobacco increased the plant's ability to survive salt stress (Pardo *et al.*, 1998). Although plant growth during salinity stress was not significantly ameliorated, plant growth, particularly root growth, was substantially improved during recovery after the salt-stress was removed. Control shoots that were grafted on to transgenic rootstock also showed significant improvement in recovery after salt removal. It is believed that this yeast calcineurin functions primarily in the root by acting on ion transport mechanisms. This effect on root ion transport then alters ion transport to the shoot and thereby affects the salt tolerance of the shoot.

There are other reports supporting the involvement of Ca signaling in salt tolerance. A Ca-binding protein is induced in salt-stressed *Arabidopsis* (Jang *et al.*, 1998). Likewise, mRNA levels of a Ca-ATPase in tomato (Wimmers *et al.*, 1992) and a Ca-dependent protein kinase in mungbean (Botella *et al.*, 1996) increase substantially after salinization. CAM induction by salinity in the halophyte, *Mesembryanthemum crystallinum*, appears to be dependent upon Ca-signaling mechanisms (Taybi and Cushman, 1999).

Is expression of the genes encoding these proteins induced because cytoplasmic Ca activities are increased or decreased? It has been generally assumed that Ca activities

are increased and this triggers the induction of some Ca-dependent proteins, presumably to further enhance the response of the biochemical pathway to elevated Ca. However, it was pointed out above that if Ca activity is increased, this response is frequently short-lived (on the order of s). Thus, the increased activity of induced enzymes comes too late. Perhaps the amounts of proteins are increased to compensate for their reduced functions when cytoplasmic Ca activity is eventually reduced by salinity?

It is interesting to note that a plasma membrane water channel can be regulated by phosphorylation in response to apoplastic water potential and cytoplasmically-relevant Ca concentrations (Johansson *et al.*, 1996). Lower external water potentials and lower Ca concentrations decrease phosphorylation of the channel. Dephosphorylation of the channel reduces water conductance through the channel (Johansson *et al.*, 1998). This may explain the role of Ca in controlling water conductance in plants described above (Sec 3.4.).

The pH gradient across the tonoplast is also affected by salinity and supplemental Ca (Martinez and Läuchli, 1993; Colmer *et al.*, 1994). Salinity causes an alkalization of the vacuole and supplemental Ca reduces this effect. It was suggested that reduced cytoplasmic Na concentrations by supplemental Ca, reduced Na/H antiport activity at the tonoplast and therefore reduce alkalization of the vacuole.

Proton extrusion is increased in mungbean roots by salinity and supplemental Ca reduces this effect (Nakamura *et al.*, 1992). The activities of the plasma membrane H^+ -ATPase and the tonoplast H^+ -ATPase and H^+ -PPase are also affected. It was suggested that elevated cytoplasmic Na activities inhibit the tonoplast H^+ -PPase, thereby stimulating the activity of the plasma membrane H^+ -ATPase. However, it should be noted that decreased cytoplasmic Ca activities also stimulate the plasma membrane H^+ -ATPase (Kinoshita *et al.*, 1995; Lino *et al.*, 1998) and it is very possible that salinity affects proton extrusion in this manner.

10.4 Summary

In summary, a large number of Na : Ca interactions occur in salt-stressed plants, particularly those in saline-sodic conditions (see Figure 2 for a hypothetical model). One of the first sites of action occurs at the external solution/root cell plasma membrane interface. These Na : Ca interactions have important effects on membrane properties and ion transport, which lead to changes in cytoplasmic Ca activity and gene expression. Na : Ca interactions can affect growth, photosynthesis, plant nutrition, water and ion transport in plants. The nature of the response will vary depending on the plant genotype.

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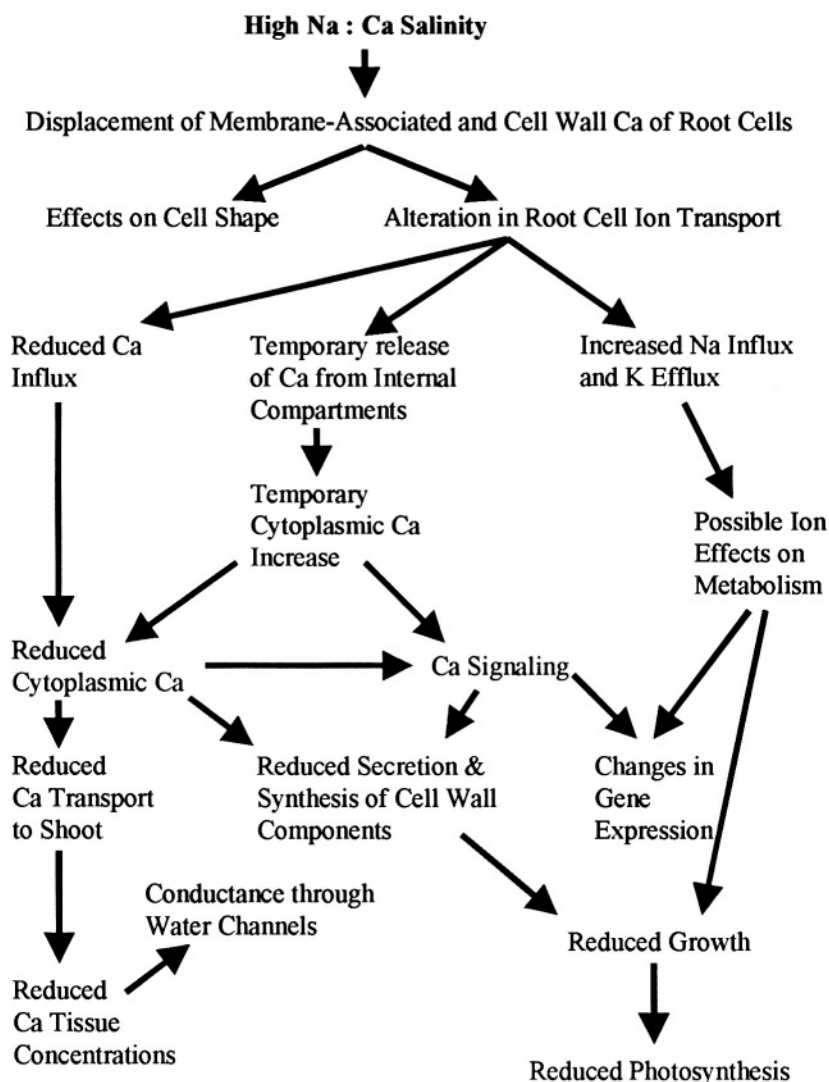


Figure 2. Schematic hypothetical model of the effects of NaCl salinity on Ca, physiology and growth.

10.5 References

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CHAPTER 11

SALINITY AND NITROGEN NUTRITION

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Abstract

Salinity can interfere with nitrogen nutrition in a direct and indirect way, usually simultaneously at several points of the assimilation pathway of the inorganic nitrogen compounds. Dependent on the plant species, there are many similarities between general osmotic stress and salt stress, even drought, but uptake of chloride can, in the case of salinity, even alleviate the mere osmotic effects. Nitrate and ammonium uptake and assimilation are inhibited by higher concentrations of Na^+ , K^+ and also Mg^{2+} to a different extent, among the anions usually sulfate is more toxic than chloride. Salinity has a tendency to shift nitrate reduction from the leaves to the roots, even in those plants that are normally 'leaf reducers'.

11.1 Introduction / general remarks

Since nitrogen is one of the most essential elements in all biological materials, changes in its nutrition and metabolism are of particular importance. These changes can lead to deficiency or to accumulation of special nitrogen compounds under salinity stress (Greenway and Munns, 1980; Stewart and Larher, 1981). Some of these compounds such as proline, glycine-betaine or other quaternary ammonium compounds have been discussed as protective agents for many years (Chapter 9). However, interference between salinity and nitrogen nutrition and nitrogen metabolism is a very complex network affecting almost all processes in plant metabolism and development. The present contribution cannot cover all these aspects but will select important points. Uptake and assimilation of inorganic nitrogen compounds will have a dominant position, changes or shifts in amino acids will be discussed for their role in responses to salinity and protection. Besides the non-infected plants of usual physiological experiments, nitrogen fixation and assimilation by symbiotic combinations with microorganisms have to be taken into account. Formation of special proteins will be considered as a part of the general changes in gene expression and plant development. Molecular genetics has become an essential tool also in this field, although not many studies have been focused yet on the regulation of nitrogen nutrition and assimilation.

Some other ions, such as sulfate, aggravate salinity effects, others mitigate them, such as calcium and increased nitrate supply, as they may do in other stress situations as well (Greenway and Munns, 1980; Pilbeam and Kirkby, 1992).

11.2 Salinity and nitrate uptake

Salinity is primarily acting in the soil, hence in the rhizosphere. The roots are the organs where nitrate but also the harmful salt ions are taken up, apart from nitrogen deposits on the leaves from polluted air with rainwater and fog, not only in industrialized or high-traffic areas. Independent of salinity, nitrate uptake by cells and tissues has been studied for many years and, as a result, has been defined as an usually biphasic process of which at least one component, the high-affinity transport follows a mechanism of cotransport of nitrate with an excess of one proton per NO_3^- (Novacky *et al.*, 1978; Ullrich and Novacky, 1981; McClure *et al.*, 1989; Mistrik and Ullrich, 1996; Crawford and Glass, 1998). Recently nitrate transporters have been characterized also at the molecular level (Forde, 2000). In a few marine algae and cyanobacteria a sodium/nitrate cotransport has been shown to exist (for diatoms Rees *et al.*, 1980, for cyanobacteria Lara *et al.*, 1993), but not in higher plants so far. The high-affinity transport system has similar characteristics in most green organisms, but the affinities for nitrate vary with the adaptation of the species to the natural environment (Ullrich, 1983, 1992). The low-affinity transport system for nitrate appeared non-saturable in some cases, but unphysiologically or better 'unecologically' high nitrate concentrations cause saturation and even a strong inhibition of the net rates of nitrate uptake, similar to salinity (Ullrich, 1983, for the green alga *Monoraphidium*; Stöhr, 1999, and Wienkoop *et al.*, 1999, for roots of higher plants). Thus, nitrate in excess is a form of salinity. Secondary active proton cotransport needs a threshold level of the membrane potential at the plasma membrane, a requirement that can be withdrawn by membrane depolarization induced otherwise. Thus concomitant uptake of cations like ammonium has been shown to transiently inhibit nitrate uptake until new equilibria with these ions have been established (Ullrich *et al.*, 1984). However, ion specificities are also involved (Crawford and Glass, 1998). The low-affinity system has not been so clearly defined in its mechanism; some authors, and for some plants, postulate for it another cotransport system, others consider it to be passive. If it is a system of anion channels (Miller and Smith, 1996), competition with chloride could directly interfere with nitrate transport. Another point that plays an important role in nitrate uptake is the very variable rate of efflux. With tracer experiments influx and efflux were shown to occur at the same time (e.g. Deane-Drummond and Glass, 1983) and apparently they can occur especially when the plasma membrane is partly depolarized by a sudden change in the ion balance, even when nitrate is fed to the plants after some time of deprivation (Ullrich *et al.*, 1990). At high external ion concentrations nitrate efflux may become a continuing process (Aslam *et al.*, 1996) resulting in exchange between nitrate and chloride or others. Miller and Smith (1996) assume a regulatory role of this efflux in stabilizing cytosolic nitrate levels.

Effects of salinity on nitrate uptake vary considerably with the plant species and with experimental conditions. Most authors report comparisons of salinity effects on transport of nitrate and ammonium. In wheat plants an inhibition by more than 50% by

60 mM NaCl of nitrate uptake rates has been observed (Botella *et al.*, 1997), whereas ammonium uptake seemed to be much less sensitive (only 8% inhibition). A weak inhibition was also seen in relative growth rates with nitrate (D.J. Pilbeam, personal communication), while there was no growth inhibition at all in ammonium fed plants. Root growth, which is generally slower in the presence of ammonium, than with nitrate, is most affected by salinity. Botella *et al.* (1994) also reported changes of kinetic data of net uptake by 60 mM NaCl. The K_m for nitrate uptake increased with salt, not V_{max} , while K_m for ammonium transport even decreased at this mild salinity. Hence, when added together, ammonium was taken up in preference to nitrate at 60 mM NaCl, but nitrate and ammonium together led to the highest yields in nitrogen acquisition. The authors claim that they found only one transport system for each of the nitrogen ions up to 1 mM. As to the ion-specific effects, Na^+ was clearly more inhibitory than chloride. With 50 mM NaCl Hawkins and Lewis (1993) observed a 40% decrease in the nitrate uptake rate, very little decrease in ammonium uptake. Calcium supply of 5 mM alleviated the salinity effects on nitrate transport by almost two thirds, while ammonium uptake remained unchanged. Part of this relief has been explained by restoring the functioning of water channels under salt inhibition (Carvajal *et al.*, 2000).

This seems to be a general observation (cf. Chapter 10) and can be explained (1) by the stabilizing effect on all membranes and (2) by a mobilization of calcium in the presence of excess Na^+ (Lynch *et al.*, 1989). A similar amelioration of nitrate uptake in barley under salinity by additional calcium was reported by Ward *et al.* (1986). In cultured cells of potato where nitrate uptake was inhibited to ca. 60% by 75 mM NaCl in the presence of already 4 mM Ca^{2+} , additional 8 mM Ca^{2+} were without effect (Hawkins and Lips, 1997), thus indicating that 4 mM are saturating for the protective effect in potato as in other plants (see also Jaenicke *et al.*, 1996).

The above mentioned results with wheat reported by Pilbeam partly differ from those with maize and wheat where salinity retarded shoot growth more than root growth (Lewis *et al.*, 1989). In these experiments a far higher sensitivity was found when grown with ammonium compared with nitrate at 50 or 80 mM NaCl. At the same time, photosynthesis and transpiration were severely inhibited. A partial relief of the salt effects could be achieved with 2 to 12 mM calcium, but only up to 60 mM salinity.

Ion specificity of salinity effects is another problem and, therefore, was approached by several authors. In wheat, polyethylene glycol (50 mM) as a non-penetrating osmotic agent proved to be similarly inhibitory to nitrate uptake but, at the same time, inhibited nitrate reductase activity in the roots to about 30%, not in the shoots (Larsson, 1992). With split-root systems the effect could be shown to be mainly local, while the non-salt-treated parts of the root showed only little influence. A much stronger inhibition by isosmotic PEG concentration than by NaCl (135 mM) was reported for *Lolium perenne* (Ourry *et al.*, 1992) where a 40% inhibition of nitrate uptake was accompanied by a 60% inhibition of nitrate reduction even in the leaves, the excess nitrate being stored in the vacuoles. The far milder effect of NaCl than of PEG was explained by the authors with uptake of chloride, which by itself seemed to be less toxic than Na^+ and could account for almost 70% of the osmoregulation. In the case of the non-permeant polyethylene glycol the cells had to achieve osmoregulation completely by synthesis of compatible solutes.

Barley is usually regarded as a relatively salt-tolerant crop species. However, salinity applied as 200 mM NaCl or Na₂SO₄ severely inhibited nitrate uptake, sulfate even more than chloride salinity (Aslam *et al.*, 1984). Anionic salinity seemed to be generally more toxic than cationic. Also in barley, increased calcium supply could prevent or at least

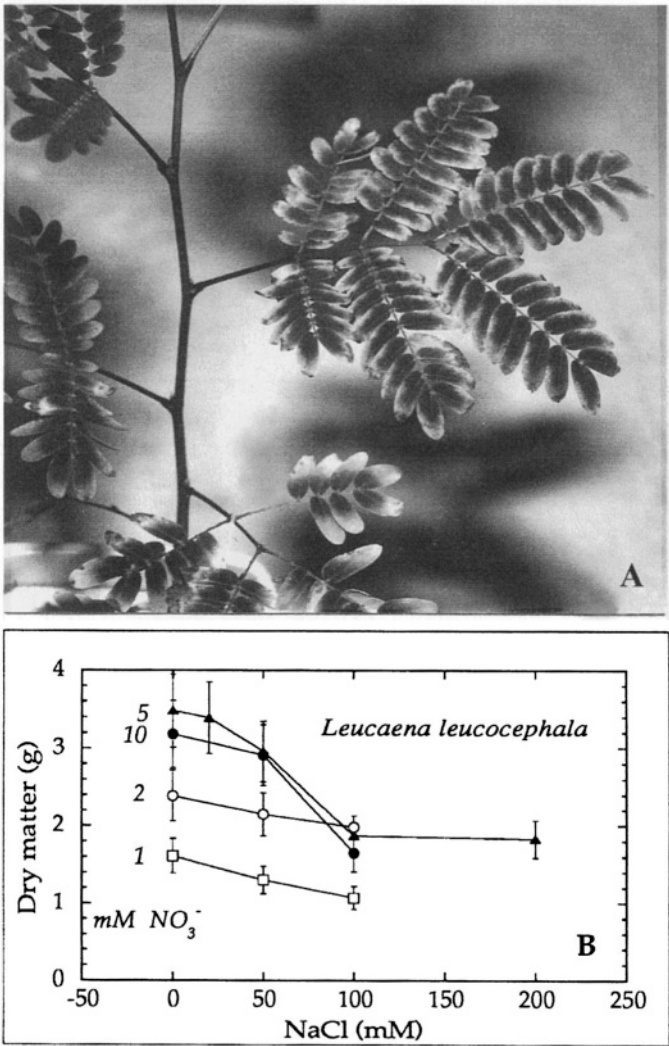


Figure 1. Effect of salinity in *Leucaena leucocephala*: (A): leaf symptoms with 1 mM nitrate and 100 mM NaCl; (B): dry matter at varied NO₃⁻ and NaCl concentrations. Plant age 42 days, treatment with the indicated media for 31 days. Calcium kept constant at 5 mM (Data from Jaenicke *et al.*, 1996).

partly relieve the inhibition of Ca^{2+} transport by NaCl. At lower concentrations (5 - 10 mM) also K^+ ameliorated NaCl inhibition of growth and protein synthesis in barley (Helal *et al.*, 1995). Recently Peuke and Jeschke (1999) reported that inhibition of nitrate uptake in barley by salt could be attributed mainly to mere osmotic effects of the salts or other solutes. However they could differentiate between non-competitive inhibition of the high-affinity nitrate uptake system by NaCl and a more competitive type of inhibition for the low-affinity system.

Other experimental plants reacted in a similar, though not necessarily the same, way with an inhibition of uptake of inorganic nitrogen. This applies also to tomato plants (Cramer and Lips, 1995), but when the root medium was perfused with air containing additional CO_2 in the water culture, nitrate uptake increased and the inhibitory effects of salinity were partly relieved, also the very strong inhibition of K^+ uptake in the presence of NaCl.

Among woody plants studied for their sensitivity of nitrogen nutrition to salt stress, fruit trees are important since they are often grown with irrigation in arid or semi-arid zones. Cerezo *et al.* (1997) investigated salinity effects in two *Citrus* cultivars known for different salt sensitivity when grown in orchards. Their calculation of nitrate uptake kinetics revealed an extremely high K_m of 282 μM in the high-affinity system and even approximate linearity for the low-affinity system. With the high-affinity system Cl^- showed a relatively clear competitive inhibition with a K_i value of 10 mM in the moderately salt-tolerant mandarin variety Cleopatra, 1.8 mM in the sensitive citrange variety Troyer. Interestingly, the low-affinity system was not salt-sensitive, when Cl^- was applied together with nitrate in the experiment, but showed strong inhibition after pretreatment with Cl^- for several hours, an indication for repression or slow inactivation. Cerezo *et al.* (1997) found a linear negative correlation between the chloride content of the tissue and the net nitrate uptake rates. Such relationships concerning the intracellular pools will be discussed in the following section.

A tropical leguminous tree often used for agroforestry and therefore of interest with respect to salinity resistance, is *Leucaena leucocephala*. Generally it showed better growth with nitrate than with ammonium and proved to be relatively salt-tolerant up to 50 mM NaCl (Jaenicke *et al.*, 1996). High supply of nitrate (10 mM) could alleviate the salt inhibition of growth and nitrate assimilation, but only at those low salt concentrations (Figure 1). When fed with low nitrate (0.5 mM) 20 mM NaCl exerted little effect on both nitrate and potassium uptake. It should be mentioned that the experiments of Jaenicke *et al.* (1996) were performed with saturating concentrations of calcium (5 mM) throughout, which also contributed to lower sensitivity to salinity (C. Sanetra, unpublished data).

11.3 Salinity and nitrate accumulation

Usually nitrate uptake is not only limited by the kinetical properties of the carrier systems, but also by the ion balances in the various cell compartments in the root or shoot tissues. Studies of such relationships have been carried out much more often with chloride or cations, for which radioactive isotopes are available, and with ions that are either non-metabolizable or more easy to follow in their metabolic pathway. Among

many trials to measure nitrate concentrations within cell compartments, the most reliable are those performed with triple-barrel microelectrodes (Miller and Smith, 1996). According to these data nitrate concentrations in the cytosol seem to be relatively constant with ca. 4 mM, but in the vacuoles they may vary considerably between a few and several hundred millimolar. The energy requirement of nitrate transport across the tonoplast can be calculated from tonoplast membrane potential differences. From this it can be concluded that the concentrations beyond 6 – 9 mM need active transport (Miller and Smith, 1996). With the actual concentrations in the vacuoles this will usually be the case. A competition between nitrate and chloride for storage has been shown in the roots of barley, a moderately salt-tolerant cereal plant (Smith, 1973). While a direct interference of chloride with nitrate at the same carrier system has not been clearly demonstrated, anion-selective channels were shown to increase the nitrate transport rate in the presence of high vacuolar chloride (Plant *et al.*, 1994). Thus, the state of the storage pools usually plays a role in uptake capacities and in efflux of ions under certain conditions (Miller and Smith, 1996). With wheat, experiments were performed explicitly in which K_m and K_i of chloride and nitrate uptake were calculated and where they showed an increase of K_m for NO_3^- in the presence of 60 mM NaCl and a slight decrease in K_m for NH_4^+ (Botella *et al.*, 1994). However, these measurements were based on the overall transport across plasma membrane and tonoplast. In most cases it may be difficult to attribute such interference to one or the other transport system and to one or the other membrane, since ion carriers for cotransport and ion channels of the two membranes may differ in their properties.

In the tropical tree *Leucaena leucocephala*, nitrate accumulation occurred at high external nitrate concentrations (10 mM) but was completely restricted to the roots, in the presence of 50 mM NaCl it was reduced to ca. 50% (Jaenicke *et al.*, 1996; Table 1). The accumulation patterns of sodium and chloride under the same conditions were rather different. Na^+ clearly accumulated in the oldest leaves, much less and only at low external nitrate in the apex and upper leaves. Cl^- was more generally distributed but its accumulation was also reduced by high nitrate, especially in the younger plant organs, thus confirming the ameliorating effect of excess nitrate against salinity stress. The distribution of K^+ was much less affected by salinity and nitrate supply. Selective salt accumulation in the oldest leaves enabled the plants to dispose of relatively high amounts of salt without losing too much of their metabolic capacities (Jaenicke *et al.*, 1996). Also in spinach and maize leaves, here in contrast to the roots, the nitrate contents were lower under salinity stress (Schröppel-Meier and Kaiser, 1988; Abd-El Baki *et al.*, 2000).

11.4 Salinity and ammonium uptake

Transport of ammonium into plant cells does not necessarily require a cotransport system, since it is a cation and is taken up through plasma membranes with a negatively charged inner side. As long as this membrane potential is maintained by ATPases cations can enter passively even when the internal concentration exceeds the external one to some extent. For these reasons and perhaps also because of special channels available, many plants take up ammonium more easily and at higher rates than nitrate. For a general review on ammonium transporters see Howitt and Utvardi (2000). At high

external ammonium concentrations and neutral or alkaline pH, ammonium was reported to accumulate to high levels of many millimolar in leaves of sugar-beet, though its transport in the xylem seems to be rather limited (Findenegg *et al.*, 1989; Pilbeam and Kirkby, 1992). For barley leaves, contents of 2–6 $\mu\text{mol g}^{-1}$ FW were reported (Schjoerring *et al.*, 1993). The process itself may lead to strong depolarization of the plasma membrane and by this seriously interferes with transport of other ions taken up via proton cotransport systems (Ullrich *et al.*, 1984). As far as channels are involved ammonium transport may interfere with K^+ transport (Pilbeam and Kirkby, 1992) and could itself be affected by the presence of higher Na^+ concentrations. Another reason for the easy transport of ammonium is that under aerobic conditions it is usually only available at low concentrations in the rhizospheres and there is not such a strong regulation by induction and repression as for nitrate uptake. Ammonium is the main natural nitrogen source only in poor and acid stands like peat bogs, heathlands or tropical and subtropical woodlands and savannas where nitrogen is a limiting nutritional factor (Pilbeam and Kirkby, 1992). As to salt sensitivity of ammonium uptake the reports differ considerably.

In most of the publications on ammonium uptake comparisons are reported between nitrate, ammonium and combined nutrition under salinity treatment. Wheat seems to be little sensitive to 60 mM NaCl with respect to total uptake and growth, but showed a preferential uptake of ammonium in the presence of salt (Botella *et al.*, 1993; 1997). According to Hawkins and Lewis (1993) in wheat the reduction in total ammonium uptake was only 16%, but with a 50% lower V_{max} . In contrast to nitrate uptake, Ca^{2+} or K^+ had no alleviating effect on ammonium uptake. On the other hand, peanut and cotton plants were distinctly more sensitive to salt when fed with ammonium than with nitrate (Leidi *et al.*, 1992). Also the N-use efficiency was lower, but the two species were different: the more salt-sensitive peanut used ammonium much better than the more salt-tolerant cotton. *Leucaena leucocephala*, similar to cotton, showed much slower growth with ammonium than nitrate and was sensitive to higher concentrations, even in the absence of salt (Jaenicke *et al.*, 1996). In soybean plants, by 100 mM NaCl only the leaf

TABLE 1. Comparison of (A), nitrate content ($\mu\text{mol g}^{-1}$ FW) with (B), nitrate reductase activity *in situ* ($\text{nmol NO}_2^- \text{g}^{-1}$ FW) in various organs of *Leucaena leucocephala*. Plants 42 days old, for 31 days in nutrient solution with: (1/0): 1 mM NO_3^- /0 mM NaCl; (1/50): 1 mM NO_3^- /50 mM NaCl; (10/0): 10 mM NO_3^- /0 mM NaCl; (10/50): 10 mM NO_3^- /50 mM NaCl. Calcium kept constant at 5 mM. (Data from Jaenicke *et al.*, 1996)

Medium		1/0	1/50	10/0	10/50
Apex	A	1.2	0.9	4.1	1.3
	B	25	35	50	50
Middle leaf	A	0.9	0.7	6.2	1.6
	B	25	25	90	75
Basal leaves	A	1.2	0.7	2.7	1.3
	B	25	28	32	33
Roots	A	6.1	1.5	52.3	24.7
	B	72	1400	530	1210

biomass was much reduced, and the sensitivity was very similar, independent of the nitrogen form supplied (Bourgeais-Chaillou *et al.*, 1992). The combination ammonium plus nitrate was the best at alleviating salt effects.

11.5 Salinity and nitrate reduction

The first metabolic step of nitrate assimilation that usually takes place within the cells is nitrate reduction, catalyzed by nitrate reductase (NR), a highly regulated enzyme (Solomonson and Barber, 1990) that often plays the role of a bottle neck for the whole process. The bulk activity of NR is located within the cytosol of the root or leaf cells under normal conditions. More recently, additional plasma membrane-bound hydrophobic forms have been discovered being located at the outer surface of the plasma membranes of both leaf and root cells and in algae (Tischner *et al.*, 1989; Stöhr *et al.*, 1995; Stöhr and Ullrich, 1997). The role of these plasma membrane-bound NR forms might be a regulatory one rather than a direct participation in nitrate assimilation. Usually the electrons for nitrate reduction are accepted from NADH or, to some extent, NADPH. The plasma membrane-bound form of roots can also use succinate as electron donor (Stöhr and Ullrich, 1997; Stöhr, 1999). The plasma membrane NR of roots shows highest activities when excess nitrate is supplied to the plants and nitrate uptake is partly inhibited. The response reminds of the inhibition by high chloride or nitrate concentrations in algae (*cf.* Ullrich, 1983). This raises the question how far NR itself is sensitive to salinity stress, by post-translational effects or by gene regulation. In the green alga *Monoraphidium braunii* the enzyme NR *in vitro* was far less salt sensitive than transport (Ullrich, 1983), but reports from higher plants vary in this respect.

The salt effects on nitrate reduction depend very much on salt concentrations and on the species investigated. Moderate salinities up to 20 or 40 mM NaCl, even if they did not visibly change plant growth, caused changes in NR activities, usually in the sense that they increased in the roots and remained little affected or unchanged in the leaves. Thus, in *Ricinus* salinity effected allocation of nitrate reduction towards a higher proportion in the roots (Peuke *et al.*, 1996). Peuke and Jeschke (1995) and Peuke *et al.* (1996) studied the distribution of inorganic and organic N and of C-compounds via the xylem in *Ricinus communis*. As expected, they found that nitrate was the main nitrogen compound of the xylem sap only in nitrate-fed plants. At low nitrate supply nitrate reduction occurred mainly in the roots, but at higher external nitrate levels increased in the leaves as well. Salinity changed this relationship by diverting a larger proportion of nitrate reduction to the roots. Assimilation of external ammonium was generally located in the roots so that amino acids were translocated in the xylem sap instead of nitrate and of malate, the latter compensating for a large proportion of the pH stat in the nitrate-fed plants.

The same shift of nitrate reduction from the shoot to the root by salinity was observed in tomato plants treated with 100 mM NaCl, although tomato is generally regarded as leaf reducer and not as root reducer (Cramer and Lips, 1995). In tobacco this was even found with 5 mM calcium without salt (Ruiz *et al.*, 1999). The salinity treatment, at the same time, shifted carbon metabolism in the roots from the synthesis of organic acids towards amino acids. This was particularly pronounced in ammonium-fed plants. In the tropical

leguminous tree *Leucaena leucocephala*, where usually NR activities of the roots are higher than those of the leaves, an even drastic increase in the roots was induced by 50 mM NaCl, while there was only little increase in the shoot, i.e. in the apex or leaves (Jaenicke *et al.*, 1996, Table 1).

The extent of NR inhibition by salinity can vary considerably. In rice, at 1 M NaCl up to 85 or 90% loss of activity was observed (Richharia *et al.*, 1997). In this case partial prevention was achieved by a pre-treatment with 0.4 mM NADH. The authors describe rice NR as a relatively salt-sensitive enzyme, but the high K_m values of 286 μM for nitrate and 4 mM for NADH cast some doubt on the preparation procedure applied in their experiments. *Lolium perenne* as a typical glycophyte of the temperate zones has a more sensitive enzyme according to Ourry *et al.* (1992) which, even at an osmolality of about 0.135 M NaCl or polyethyleneglycol, was inhibited by about 60%.

Differential studies on salinity effects on NR and nitrite reductase (NiR) activities have been reported for *Sorghum vulgare* (Rao and Gnanam, 1990). In this plant after salinization of the seedlings *in vitro* activity of NR was much more inhibited (to 35%) than that of NiR (only to 67% by 1 M NaCl). In the assay mixture salt was again more noxious to NR. Comparison of different salts revealed that sulfate was more inhibitory than chloride, and KCl caused far stronger inhibition than NaCl, even at much lower concentrations. Comparison between Cl^- , SO_4^{2-} and polyethylene glycol (PEG) with tomato seedlings resulted in the strongest inhibition of both nitrate uptake and NR activity by Cl^- , although growth was most sensitive to PEG (Flores *et al.*, 2000). As to the effect of other cations, magnesium was studied by Lillo (1994) with crude extracts from leaves of various plants. Magnesium is usually required by kinases and phosphatases and is known to be accumulated to several millimolar in the chloroplasts during photosynthesis. The complete NR proved to be partly inhibited in crude extracts by 5 mM Mg^{2+} , especially when extracted in the dark. The same could be shown for the NADH/cyt *c* partial reaction, while a methylviologen-driven nitrate reduction was insensitive to that concentration. ATP when added to the extracts from light experiments aggravated the inhibition to the level of the dark experiments.

An even more detailed analysis of the sensitivity of the partial reactions to ionic strength, in this case produced by concentrated Mops and Mes buffers, was given by Barber *et al.* (1989). In contrast to NADH-NR, NADH-ferricyanide reductase and NADH-cyt *c* reductase were completely insensitive to ionic strengths up to 300 mM. Together with a decrease in V_{\max} , K_m increased with ionic strength from 13 μM at 50 mM to 18 μM at 200 mM Mops buffer, indeed rather low values, since the usually applied 5 mM phosphate buffer gave a K_m of 93 μM . K_i for Cl^- resulted as 176 mM showing a relatively weak interference. Apparently the interaction site of Cl^- was Mo(V) in the terminal domain of the enzyme.

This again leads to the question how NR is regulated, for which several pathways have been elucidated. Besides a regulation by gene expression that is certainly involved in adaptation to salinity, post-translational regulation has become known in recent years. The main mechanism of post-translational regulation proceeds via phosphorylation/dephosphorylation of the NR protein at several serine residues and the stabilization of the inactive phosphorylated forms by binding to 14-3-3-proteins (Kaiser and Huber, 1994; MacKintosh *et al.*, 1995; Moorhead *et al.*, 1996; Athwal *et al.*, 1998). There are,

however, other mechanisms that can lead to partial or even total inhibition of NR and seem to be completely independent of phosphorylation. One of them is using the redox state or binding of cyanide as postulated many years ago for NR *in vivo* in algae (Pistorius *et al.* 1979). Recently Munjal *et al.* (1997 a, b) reported on two ways of inactivation and reactivation of NR from wheat. Ferricyanide when added to leaf extracts could reactivate this NR within two days, with NADH recovery was even slower, a recovery discussed by the authors as interference with reversible binding to NR of a proteinaceous inhibitor. This mechanism was obviously independent of phosphorylation, which Munjal *et al.* (1997) could provoke only with 10 mM ATP thus obtaining a reduction in activity to about 40%. Regulation by gene expression and inhibition by salinity was shown by different levels of NR-mRNA in maize (Abd-El Baki *et al.*, 2000).

Another group of more recent articles deals with the involvement in regulation of the molybdenum cofactor (molybdopterin, MoCo) located in the terminal domain of NR. In plants and animals several enzymes contain this cofactor, aldehyde oxidase, xanthine oxidase, only in plants e.g. indolaldehyde oxidase and NR. In barley salinity, or even ammonium instead of nitrate nutrition, stimulated synthesis of molybdopterin complexes in general (Omarov *et al.*, 1998). This additional synthesis mainly favored aldehyde oxidase in the roots, salt and ammonium together producing the highest activities, while NR activity decreased. Hence, there seems to be a common pool of molybdenum cofactor whose distribution among the respective enzymes is regulated by the nutritional nitrogen form and salt stress. Interestingly, the leaf aldehyde oxidase remained almost unaffected by these conditions (Omarov *et al.*, 1998). Similar results as for barley were reported for the more salt-sensitive *Lolium multiflorum* (Sagi *et al.*, 1997), where MoCo could be assayed by complementation of NR with the apoprotein of a MoCo-free *nit-1* mutant of the fungus *Neurospora crassa*.

11.6 Salinity and ammonium assimilation

Less is known so far about the responses of ammonium assimilation to salinity in the absence of nodules with dinitrogen fixation. In a comparative study on all the enzymes involved in nitrogen assimilation, Bourgeais-Chaillou *et al.* (1992) reported experiments with soybeans and different N sources (nitrate, ammonium or ammonium plus nitrate) and with 100 mM NaCl. The biomass of roots and stems was not altered by all these treatments, but the leaf biomass and the protein content of both nitrate and ammonium plants was much lower in the presence of NaCl. As in *Leucaena* and other plants, the NR activity of roots increased by salt, while glutamine synthetase (GS) became less active. NiR, GS, glutamate synthase, phosphoenolpyruvate carboxylase and Rubisco were less affected by salt treatment in plants fed with NH_4NO_3 than with NO_3^- or NH_4^+ .

In peanut (*Arachis hypogaea*) and cotton (*Gossypium hirsutum*) ammonium nutrition resulted in a less efficient use of nitrogen under saline conditions (Leidi *et al.*, 1992). To some extent this may be due to different ion balances, especially lower K^+ levels, but in addition ammonium is almost exclusively assimilated in the roots, whereas nitrate can be transported to the photosynthetically active leaves as well. While peanut as a salt-sensitive species could reach comparable nitrogen contents with ammonium and showed

less salt inhibition with ammonium, cotton, though usually regarded as a moderately salt-tolerant plant revealed the lower efficiency. Similar to cotton, also *Leucaena leucocephala* showed considerably lower growth with ammonium and even a sensitivity at high concentrations (Jaenicke *et al.*, 1996), but salinity experiments with ammonium are lacking.

11.7 Effects of salinity on amino acid patterns

Since osmolytes and compatible solutes are dealt with in Chapter 9 of this volume, only some special aspects concerning shifts in amino acid metabolism and accumulation will be discussed in this paragraph.

As mentioned before, in many plants total nitrogen content was not seriously affected by low or moderate salinity, even if NR was partly inhibited. In these cases usually the activity of NR in control plants was in excess so that a decrease by salinity did not become limiting. But there was also that tendency to shift nitrate reduction mainly to the root and towards transport of amino acids instead of nitrate in the xylem stream. These changes together with reduced growth, especially in the leaves and with new osmotic balances, as required with additional ion deposition, will affect amino acid patterns (Treichel, 1975; Greenway and Munns, 1980). Amino acids are often the main so-called 'compatible solutes' and 'osmoprotectants' under conditions of stress by salinity, drought and others (see Chapter 9). Accumulation of amino acids may help the plants to keep sound water relations and, at the same time, to control uptake, exclusion or release of the toxic inorganic ions, but the mechanisms of such regulation processes are still widely unknown.

In earlier work the pools of free amino acids and protein amino acids were investigated, *e.g.* in the typical glycophyte *Pisum sativum* (Bar-Nun and Poljakoff-Mayber, 1977) at up to 120 mM NaCl and, for comparison, in *Tamarix tetragyna*, a halophyte of semiarid and arid zones, with up to 480 mM NaCl. Proline accumulated in *Tamarix* with an increase up to high salinity, valine and leucine only at moderate salt concentrations. Aspartic acid, proline and alanine accumulated in *Pisum*. Others decreased under salt stress. These data already show the very complex and species-dependent response of amino acid levels, a problem found throughout the whole literature. Generally the quantitative pattern of amino acids and its changes by salinity could not account for osmotic equilibria with the external salt concentrations. Hence the main role of the free amino acids may not be to balance ionic strength but rather to specifically protect membranes and sensitive proteins against the effects of toxic ions.

Sporobolus virginicus, a grass from salt-marshes and sea-shores, which grew best at one fifth of the salt concentrations of sea water, accumulated proline by 15-fold under salt treatment (Naidoo and Naidoo, 1998). Also here, the overall concentrations of up to 17 μmol proline g^{-1} DW in roots and up to 23 in the shoots can only be regarded as an essential contribution to osmotic balance when accumulated locally in certain cell organelles.

In the moderately salt-tolerant cereal barley (*Hordeum vulgare*) (Wyn Jones and Storey, 1978) with increasing salinity or osmotic stress by polyethylene glycol, both proline and

glycine-betaine accumulated. Was the salinity level increased gradually, glycine-betaine was the preferred amino acid, upon salt or osmotic shock proline levels were far in excess of those of glycine-betaine. The relative increase was always greater for proline than for glycine-betaine. The low absolute contents of the two solutes even with osmotic or salt stress were explained by the authors in the way that both solutes were widely excluded from the vacuoles, hence attaining much higher concentrations in the cytoplasmic phases of the cells, while the vacuoles mainly accumulated salt.

For maize a glycine-betaine producing and a deficient mutant (*Bet1/Bet1* and *bet1/bet1*) were compared with respect to salt tolerance (Saneoka *et al.*, 1995). The deficient plants were more salt-sensitive (150 mM NaCl). The authors conclude that the *bet1* gene and thus glycine-betaine plays a key role in osmotic adjustment in maize.

Intracellular distribution of glycine-betaine and its response to salt was studied in spinach (Robinson and Jones, 1986). In their experiments with 200 mM NaCl, the overall glycine-betaine content rose to about six-fold under salt stress, while choline or proline remained almost unchanged. However, in isolated chloroplasts, glycine-betaine even reached concentrations up to 300 mM, i.e. 20-times those of the leaf as a whole. In such cases, glycine-betaine can account for a great proportion of the osmotic protection required.

With respect to chloroplasts a quite different response of solute formation to salinity stress was reported for spinach leaves (Schröppel-Meier and Kaiser, 1988). For salinity the authors used either NaCl or NaNO₃ up to 300 mM. The relatively salt-tolerant spinach was compared with pea where comparable responses occur at much lower salt concentrations than with spinach.

In *Coleus blumei*, a rather salt-sensitive plant, amino acid levels were followed after salinity treatment with 60 mM NaCl (+12 mM CaCl₂) for one month (Gilbert *et al.*, 1998). Growth was reduced to about half the size of the control plants and there was a further relative decrease in protein content. The levels of most free amino acids rose severalfold within the first 5 to 10 days of the salt treatment, but most of them fell back to almost normal within 10 to 20 days. Proline remained at the same level as in the control plants. Pulse-chase experiments with ¹⁴CO₂ revealed that most of the amino acid increase was due to new formation of the C-skeletons by photosynthesis, not to protein hydrolysis.

As mentioned earlier, in various plants salinity stress causes shifts of nitrate reducing activity from the leaves to the roots. In tomato, together with the strong inhibition of nitrate uptake by the roots, less nitrate and more amino acids were transported in the xylem and the metabolism in the tissues shifted from free organic acids towards amino acid formation (Cramer *et al.*, 1995). This was also found in ¹⁴C-experiments with higher CO₂ supply in root aeration (Cramer and Lips, 1995).

Several authors focused their research upon the role of metabolic shifts and on the effects of the products as protecting agents against salinity stress. When supplied externally to the roots of rice plants in hydroponics (Garcia *et al.*, 1997), proline had either no effect at all or even exasperated growth inhibition by salt stress and increased the expression of a marker gene, *salT*, which indicates stress, whereas trehalose, also a natural product in rice, reduced Na⁺ accumulation in the tissues, *salT* expression and the

growth inhibition. Up to 10 mM, trehalose prevented chlorophyll losses and root tissue degeneration, and even stimulated growth. Similarly, in soybean proline accumulated particularly in a salt-sensitive cultivar but only as an effect of considerable salt damage (Moftah and Michel, 1987). Like various others, these studies cast doubt on a proposed general role of proline as a protectant against stress.

For the moderately salt-tolerant *Sorghum bicolor* Colmer *et al.* (1996) reported a very pronounced induction of proline accumulation, especially in the root tips, by 150 mM NaCl. This effect was multiplied by high Ca^{2+} (5 mM compared with 0.5 mM) producing proline levels 50 times those of the controls (4 times at 0.5 mM Ca^{2+}) within two days. However, the authors report more distinctly about the mitigating effect of calcium on the K^+/Na^+ ratio than about the role of proline under these circumstances.

A comparison between a salt-sensitive and a more salt-tolerant variety of tomato (Aziz *et al.*, 1998) again gave ambiguous results. Proline, polyamines and tyramine were much more accumulated in the salt-sensitive than in the tolerant cultivar. Proline accumulation started already after 4 h of salt treatment and was the quantitatively strongest effect.

11.8 Salinity and nitrogen nutrition in nodulated Legumes

There are several reports on salinity effects on nodulated *Fabaceae* but with quite different aims and results. The research was focused either on nitrogen fixation itself or on the changing pattern of assimilation products compared with rhizobial activities under salt stress.

In seedlings of *Lupinus albus* as a salt-sensitive plant intensively nodulated with a *Rhizobium* strain (Jeschke *et al.*, 1986), salt treatment remarkably increased the total amino acid levels and some individual amino acids in the xylem sap, among them especially asparagine and aspartate, and valine at a lower concentration level. These changes were even more pronounced in samples of phloem sap, where serine joined the enhanced amino acids. Proline was not involved and scarcely detectable under those conditions, another indication that it does not necessarily play the dominant role as a protectant and compatible solute. How rhizobial activity responded to the salt treatment in lupin was not reported.

With respect to compartmentation and to the contribution by plant roots and bacteroids a more detailed analysis was performed with alfalfa (*Medicago sativa*) (Fougère *et al.*, 1991). Asparagine, glutamate, alanine, γ -aminobutyrate and proline were the major amino acids in the cytosol and bacteroids of the nodules, serine and glutamine were equally present in the normal root tissues. After two weeks with 150 mM NaCl, most levels of organic acids were depressed, while lactate became dominant, especially in the bacteroids. Among the amino acids proline accumulated most, up to eight- or twelve-fold. Also asparagine was greatly enhanced and may have played an osmoregulatory role in bacteroids. Among the carbohydrates the pool sizes of pinitol increased very much, trehalose remained on a low level in roots and in the nodules and, hence, could not play a significant role as a protectant.

Salinity effects on N_2 fixation, assayed as acetylene reduction as usual, were included in some investigations with the salt-sensitive *Vicia faba* combined with a relatively salt-tolerant strain of *Rhizobium leguminosarum* biovar. *viciae* (Cordovilla *et al.*, 1994, 1996, 1999). In these plants, by salinity up to 100 mM NaCl, total nitrogen was even more depressed than growth. Moreover, nitrogenase (acetylene reduction) was more sensitive to low salt concentrations than glutamine synthetase and NADH-glutamate synthase of the host plants. Addition of 8 mM KNO_3 increased the salinity tolerance of *V. faba* even at 100 mM salt, although it reduced N_2 fixation (acetylene reduction) and the ammonium metabolizing enzymes in the nodules (Cordovilla *et al.*, 1996). Apparently NADH-glutamate synthase was the limiting enzyme in the nodules under salt stress. Again in these experiments there was no relationship between proline content and salt tolerance of the plants.

A strong inhibition of acetylene reduction by 50 mM NaCl was reported for nodulated *Pisum sativum* (Delgado *et al.*, 1993). In the nodules of this combination, activities of cytosolic phosphoenol pyruvate carboxylase (PEPC) and bacteroid malate dehydrogenase (MDH) were considerably enhanced. At the same time, respiration of the bacteroids was depressed, together with a shift towards fermentation. Salt caused a decrease also in the cytosolic proteins, especially of leghemoglobin, which is in line with the lower respiration rates and with the higher levels of lactic acid reported by Fougère *et al.* (1991). A very similar stimulation of PEPC and MDH by 50 mM NaCl, but only for a shorter time, was reported for *Cicer arietinum* (chick-pea) by Soussi *et al.* (1998, 1999). Acetylene reduction was rather sensitive, more sensitive than photosynthesis. According to these experiments, the accumulation of the so-called compatible solutes accompanying the salt stress is regarded as a consequence of salt damage rather than a protective strategy. In *Pisum sativum* and *Cicer arietinum* a greater mass of nodules could compensate for lower activities in salt-tolerant rhizobia strains (Cordovilla *et al.*, 1999).

A rather detailed analysis of salt effects within the nodules and the separate compartments (cytosol of nodule cells, peribacteroid space and bacteroids), similar to that of Fougère *et al.* with alfalfa, was given for nodulated *Vicia faba* (Trinchant *et al.*, 1998). 100 mM NaCl for two weeks effected a loss of acetylene reduction by ca. 50%, but the amino acid content of the nodules greatly increased, mainly in the cytosolic compartment. Asparagine accounted for more than 50% of the total amino acids in all compartments, but the levels were far lower in the bacteroids and the peribacteroid space. Proline showed the highest relative increase, especially in cytosol and peribacteroid space, but only there and under salt the contribution to total amino acids exceeded that of asparagine. From these data the authors, in contrast to Cordovilla *et al.* (1994), conclude that proline may play a role as an osmoticum and in stabilizing membrane phospholipids.

The controversial results and views about the role of proline in protection against salt also became apparent in recent genetic investigations (Liu and Zhu, 1997; Zhu *et al.*, 1998) with the highly salt-sensitive *sos* mutants of *Arabidopsis thaliana* in comparison with the wildtype. The 20-times more salt-sensitive *sos1*-mutant accumulated much more proline and revealed a higher induction of the *P5CS*-gene whose protein (Δ^1 -pyrroline-5-carboxylate synthase) catalyses the rate-limiting step in proline synthesis. The *sos* mutants also have a defective high-affinity K^+ uptake system that

causes K^+ deficiency and a special sensitivity to Na^+ or Li^+ salinity, not generally to osmotic stress. In conclusion, again in this case, the overproduction of proline by salinity in the *sos* mutants is not directly related to salt tolerance, but may even be more pronounced in salt-sensitive species or strains.

11.9 Salinity and protein synthesis, molecular aspects

Salinity effects on protein synthesis are not restricted to nitrogen nutrition and, therefore, are discussed also in other chapters of the volume. Changes in protein synthesis play a role in many regulation processes as seen in the previous paragraphs of this chapter, although this role has not been studied in many of the experiments so far.

Most of the literature on molecular biology and on the synthesis of special proteins under salinity stress does not at all or only marginally deal with responses to nitrogen nutrition. In an older report (Helal *et al.*, 1975), general effects of NaCl on $^{15}NO_3^-$ incorporation and distribution, protein formation and labelling were not impaired by up to 120 mM NaCl in barley, in spite of an inhibition of $^{15}NO_3^-$ uptake. Addition of KCl alleviated the negative effects of NaCl in general and further stimulated protein labelling, indicating that in this case Na^+ was the more toxic ion.

More focused on protein synthesis, but on drought instead of salt effects, were studies with rice, where a salt-sensitive cultivar with *salT* gene induced 8 proteins. For one of them a cDNA clone with an open reading frame was selected (Claes *et al.*, 1990). The salt RNA rapidly accumulated in all parts of the plants except the leaf laminae, and was inducible with ABA, polyethylene glycol, 1% NaCl or 1% KCl.

Several papers concentrated mainly on the induction and molecular mechanisms of salt adaptation and CAM development in *Mesembryanthemum crystallinum* (Vernon *et al.*, 1993; review by Bohnert *et al.*, 1995, see Chapter 17). Four genes were activated by salinity and other stress factors, but these genes unspecifically produced various proteins including those involved in the synthesis of choline, betaine groups and the cyclic amino acids ectoine and hydroxyectoine (Bohnert *et al.*, 1995). For proline accumulation as a response to salinity in a highly salt-sensitive *Arabidopsis thaliana* mutant (*sos1*), far more than in the wildtype (Liu and Zhu, 1997), expression of *P5CS* (see 11.9) was found to be responsible, at least for an essential contribution. A proline-rich cell wall protein encoded by a special DNA sequence and post-transcriptionally regulated was reported for alfalfa as a response to salt stress (Deutch and Winicov, 1995). In two transgenic lines of *Nicotiana plumbaginifolia* exhibiting reduced GS production mainly in the phloem, the proline content was generally much lower. These plants showed clear stress symptoms after salt treatment thus indicating a particular role of phloem GS in stress adaptation (Brugière *et al.*, 1999).

Molecular genetics of salt stress tolerance has been studied extensively with yeast (Serrano and Gaxiola, 1994; Chapter 21), but in some cases these genes from yeast were transferred to tomato plants where salt tolerance was markedly increased (Serrano and Gaxiola, 1994; Arrillaga *et al.*, 1998).

Another interesting approach to protein synthesis and protein modifications as a response to salinity was achieved with *Nicotiana sylvestris*, where a correlation between

previous heat treatment and salt tolerance was revealed (Kuznetsov and Roshchupkin, 1994; Kuznetsov and Shevyakova, 1997). Synthesis of heat-shock proteins accompanied this salt tolerance acquirement but it appeared that mainly the selective phosphorylation of some newly-formed proteins was responsible for heat and salt tolerance, and this was followed by proline accumulation. In cotton (*Gossypium hirsutum* and *G. barbadense*) salt tolerance was increased after a release of ethylene, accompanied by accumulation of proline and putrescine and osmotin-like proteins (Kuznetsov *et al.*, 1993). General reviews on the molecular basis of dehydration tolerance were published by Ingram and Bartels (1996) and on molecular mechanisms of ion homeostasis during salt stress by Serrano *et al.* (1999).

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11.10 References

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CHAPTER 12

PRESSURE PROBE MEASUREMENTS OF THE DRIVING FORCES FOR WATER TRANSPORT IN INTACT HIGHER PLANTS: EFFECTS OF TRANSPIRATION AND SALINITY.

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Abstract

Effects of salinity on the driving forces for water transport in higher plants can only be accurately determined when the “common” reaction pattern of the plant in the absence of salinity stress is known. We will demonstrate that in the absence of salinity xylem and turgor pressures react very sensitively to changes in the environmental conditions (especially to light intensity changes), exhibiting transpiration-related, highly dynamic reaction patterns which can be recorded by the direct, minimal-invasive xylem and turgor pressure probe techniques. The magnitude of xylem and cell turgor pressure responses to salinity clearly depends on the transpirational status of the plant, at least for various grass species. Interestingly, application of HgCl_2 to the root amplifies the effects of salt stress, suggesting that water and solute flows into and out of the root are disturbed by this treatment.

13.1 Introduction

Many attempts have been made to elucidate the force-flow pattern of long-distance water and solute transport in higher plants under various environmental and culture conditions. Unfortunately, no satisfactory biophysical methods have existed in the past to accurately and directly measure the flow and forces involved in xylem transport from the roots to the leaves. Most of the current methods used for measuring xylem flow and pressure (frequently on detached plant material) are clearly inadequate because of the uncertainty of what is measured and due to the complex coupling phenomena between the xylem and the tissue that may considerably change when excised plant organs or tissues are investigated.

The cell turgor and xylem pressure probe technique introduced by Zimmermann *et al.* (1969) and by Balling and Zimmermann (1990), respectively, as well as its recent

modifications (Wegner and Zimmermann, 1998) provide a solution to overcome these problems and to determine minimal-invasively the electrical and hydraulic characteristics of intact, transpiring plants.

These measurements have made it necessary to revise our view on the forces involved in water ascent in higher plants and tall trees (Zimmermann *et al.*, 1993 a, b, 1994 a, b, 1995 a, b, 2001; Shackel, 1996; Melcher *et al.*, 1998; Thürmer *et al.*, 1999; Schneider *et al.*, 1999, 2000; Wagner *et al.*, 2000; Wistuba *et al.*, 2000).

In this review article, the authors will attempt to elaborate on some physical aspects of the fundamental processes employed in xylem transport of glycophytes and halophytes revealed by the probe technique. Emphasis is particularly given to the response of xylem pressure and cell turgor pressure of the tissue cells upon (diurnal) changes in transpiration as well as upon short-term salt stress.

Despite considerable progress in the last few years, this field of study is still in its infancy. However, the data presented here reveal the gaps in our knowledge and, thus, may provide a guideline for new technical developments and future experiments.

13.2 What negative pressure values can be expected in the xylem?

There is a bulk of evidence that the xylem and the surrounding tissue cells behave like a Hepp-type osmometer (Mauro, 1965, 1981; Balling *et al.*, 1988; Balling and Zimmermann, 1990). Due to the rapid water exchange times of the tissue cells (some 10 s; Zimmermann, 1989; Steudle, 1989, 1992; Moore and Cosgrove, 1991; Malone, 1993) one can always assume water equilibrium between the two compartments. Thus, the chemical potential of water (μ) of both compartments must be equal at a given height (h) if electrical terms are neglected:

$$\mu_x(h) = \mu_c(h) \quad (1)$$

and correspondingly (Pickard, 1981; Benkert *et al.*, 1995):

$$Mgh + V(P_x - P_{am}) + RT \ln a_x = V(P_c - P_{am}) + RT \ln a_c \quad (2)$$

where the subscripts x and c denote the xylem conduit and the tissue cell compartment, respectively; M = molecular mass; g = gravitational constant; h = distance from the ground; V = partial molar volume of water; R = gas constant; T = absolute temperature; a = activity of water; P = hydrostatic pressure in absolute values; P_{am} = ambient pressure; $P_c - P_{am}$ = turgor pressure.

If the reduction of the water activity is envisaged exclusively by osmotically active (low molecular weight) solutes, Eq. 3 holds:

$$\rho gh + (P_x - P_{am}) - \pi_x = (P_c - P_{am}) - \pi_c \quad (3)$$

where π is the osmotic pressure.

The equations demonstrate that the steady state hydrostatic pressures in the xylem and in the cells are independent of the ambient pressure as revealed by hyperbaric chamber experiments (Balling and Zimmermann, 1990; but see the comments of Passioura, 1991).

Furthermore, Eqs. 2 and 3 also show that the xylem pressure is determined by the “water potential” of the cells, but not *a priori* by the transpiration rate (Balling and Zimmermann, 1990; Thürmer *et al.*, 1999; Schneider *et al.*, 1999). When transpirational loss of water occurs and is not compensated by a corresponding water uptake through the root, a new water equilibrium state will be established by an appropriate decrease of the xylem and cell turgor pressure. According to Eq. 3, a linear change in both parameters should occur if the osmotic pressure within the cells and the vessels remains constant (Thürmer *et al.*, 1999). Otherwise (depending on the magnitude of the volumetric elastic moduli of the adjacent tissue cells), the corresponding change in the xylem pressure should be slightly larger than the change in turgor pressure (Schneider *et al.*, 1999).

Eq. 3 predicts that the minimum **stable** negative xylem pressure which can exist in a vessel is reached when the turgor pressure becomes zero. Assuming for example an average turgor pressure value of about 0.5 MPa¹ before the onset of transpiration (with xylem pressures near the atmospheric value; Thürmer *et al.*, 1999), this implies that the minimum stable xylem pressure should assume values around -0.4 MPa provided that both parameters change in a 1 : 1 relationship upon transpirational variations. If the relationship between turgor pressure and xylem pressure changes is less than 1 : 1 (e.g. 0.7 : 1 in a drought-resistant resurrection plant; see Schneider *et al.*, 1999), the xylem pressure can assume somewhat lower minimum stable values from a thermodynamical standpoint (which does not take into account the increase in cavitation probability with increasing tension; see Mauro, 1981). Below the lowest stable xylem pressure values predicted by Eq. 3 no equilibrium between the vessels and the cells can be established, with the consequence (and in agreement with xylem pressure probe measurements) that the tension continuously and very rapidly increases until cavitation occurs.

Even though our knowledge is rather poor about pressure profiles in the stele region of a root for transport, the finding of radial, inwardly increasing turgor and osmotic pressure gradients in the root cortex of intact, transpiring halophytes and glycophytes (of up to 0.5 MPa; Zimmermann *et al.*, 1992; Rygol *et al.*, 1993) may be important in the temporary establishment of relatively large negative xylem pressures when the plants are subjected to high transpiration rates as well as to drought and salt stress.

13.3 Probe techniques *versus* conventional techniques

In order to reveal the mechanisms of long-distance water transport in transpiring plants in response to drought and salt stress, the hydrostatic and osmotic forces operating in the xylem and tissue compartment (see Eq. 3) must be known.

¹ Note that all xylem pressure values are quoted as absolute pressures (atmospheric pressure = +0.1 MPa); 0.1 MPa tension corresponds to a xylem pressure of 0.0 MPa (vacuum). In contrast to the values of the xylem pressure, the turgor pressure of the cells is defined relative to the ambient atmospheric pressure (see Eq. 2).

The pressure probe technique allows continuous measurements of the xylem pressure and of the cell turgor pressure in intact, transpiring plants on the level of a single vessel and tissue cell, respectively. In addition, cell sap sampling by means of the turgor pressure probe in combination with pico- and nanolitre osmometry (Tomos *et al.*, 1994) enables the determination of the osmotic pressure within the cells and, thus, the estimation of the activity of water (i.e. the osmotic pressure according to Eq. 3) in the xylem vessels or, generally speaking, in the apoplastic space (Thürmer *et al.*, 1999; Schneider *et al.*, 1999).

13.3.1 CELL TURGOR PRESSURE PROBE

The turgor pressure of individual tissue cells of intact (transpiring) plants can accurately be determined by means of the turgor pressure probe (Figure 1A; see also Pritchard *et al.*, 1989; Steudle, 1989, 1993; Spollen and Sharp, 1991; Zimmermann *et al.*, 1992; Rygol *et al.*, 1993; Fricke, 1997; Schneider *et al.*, 1997 b, 1999; Franks *et al.*, 1998; Thürmer *et al.*, 1999). In contrast, the plasmolysis technique is restricted to excised tissues (Lee-Stadelmann and Stadelmann, 1989). Moreover, the latter technique only provides mean values of the cell turgor pressures within a tissue, since turgor (and osmotic) pressure gradients in roots and stems, which are obligatory for transpiring halophytes and glycophytes (Zimmermann *et al.*, 1992; Rygol *et al.*, 1993; Schneider *et al.*, 1997 b), collapse upon tissue excision (Zimmermann *et al.*, 1992; Rygol *et al.*, 1993).

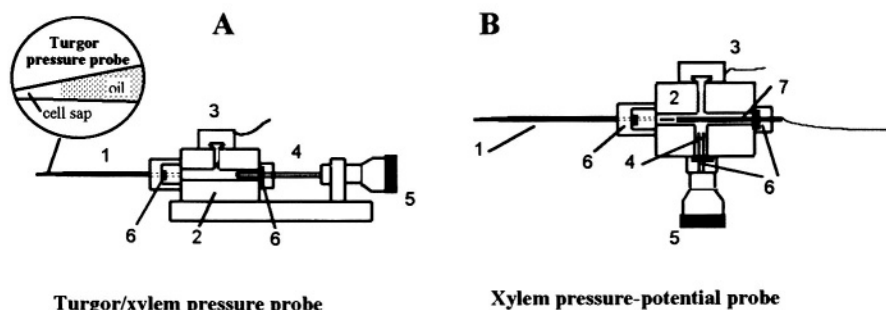


Figure 1. Schematic diagram of the turgor and xylem pressure probe (A) and the xylem pressure-potential probe (B). The principles of pressure sensing by the xylem and turgor pressure probe are identical except that the turgor pressure probe is filled with oil (see inset in A) whereas the xylem pressure probe contains degassed and deionized water. The numbers denote: 1 = microcapillary; 2 = perspex chamber; 3 = pressure transducer; 4 = metal rod; 5 = micrometer screw; 6 = pressure-tight rubber seals; 7 = Ag/AgCl electrode. For further explanations, see text.

The principle and the possible pitfalls of the cell turgor pressure probe are described in detail elsewhere (Zimmermann *et al.*, 1969; Zimmermann *et al.*, 1992; Rygol *et al.*, 1993; Murphy and Smith, 1998). Briefly, a single cell is impaled with an oil-filled microcapillary (see inset of Figure 1A). The turgor pressure inside the cell is transmitted via the oil to a pressure transducer mounted in the perspex chamber of the probe. The pressure transducer transforms (mechanical) pressure signals into proportional voltage signals. Interference effects arising from compression of the oil and temperature

fluctuations in the system are excluded by means of an accurate adjustment of the cell sap/oil boundary in the tip of the microcapillary (see inset of Figure 1A). This is achieved by appropriate displacement of a metal rod integrated into the pressure probe via pressure-tight seals. The visible boundary between oil and sap (which can be kept in a fixed position) enables the accurate measurement of the kinetics of pressure changes, in addition to equilibrium pressures.

13.3.2 XYLEM PRESSURE PROBE

The cell turgor pressure probe can also be used to monitor the pressure in the xylem (Zimmermann *et al.*, 1993 a 1994 b; Figure 1B). However, for long-term measurements in a xylem vessel it is advantageous to use deionized and degassed water (Balling *et al.*, 1988; Balling and Zimmermann, 1990; Zimmermann *et al.*, 1991, 1993 a, b, 1994 a, b, 1995 a, b; Benkert *et al.*, 1991, 1995; Benkert, 1994; Zhu *et al.*, 1995; Schneider *et al.*, 1997 a, b, 1999; Melcher *et al.*, 1998; Wegner and Zimmermann, 1998; Thürmer *et al.*, 1999; Wistuba *et al.*, 2000) instead of the silicone oil because of the reduced probability of cavitation and contamination of the xylem sap (Figure 1A). Manual or automatic placement of the probe in a xylem vessel of an intact plant is detected by a rapid drop of the pressure from (above-) atmospheric to sub-atmospheric, positive or negative values during advancement of the probe through the tissue (see below). The punctured vessel becomes stained when a dye was pre-dissolved in the water of the microcapillary (Figure 2).

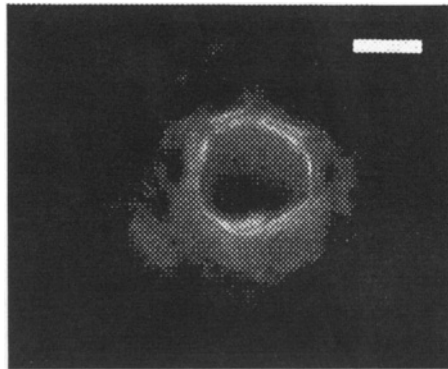


Figure 2. Typical fluorescence microscopy image of a xylem vessel after insertion of the microcapillary of the probe. The microcapillary was filled with 0.1% Calcofluor White dissolved in degassed distilled water. The photograph (bar = 20 μm) was taken about 15 min after probing of the vessel close to the insertion site. Excitation wavelength 365 nm.

In contrast to current indirect methods used for the determination of “xylem pressures” (see below), the xylem pressure probe has been critically evaluated in various model experiments (Balling *et al.*, 1988; Schneider *et al.*, 1997a; Thürmer *et al.*, 1999). These studies have shown that the probe can read absolute negative pressures at least down to about -1 MPa (Zimmermann *et al.*, 1995 b; Thürmer *et al.*, 1999). Such pressure values

can be realized in the laboratory provided that the development of tension is quite slow and that highly vibration-free conditions are chosen (Thürmer *et al.*, 1999).

Nevertheless, measurements in herbaceous plants and in leaves of tall trees have demonstrated that the tension in the xylem vessels is substantially less than the highest tensions measurable with the probe. Negative pressures down to about -0.6 MPa could be generated occasionally in vessels of herbaceous plants subjected to drought or salt stress or when the vessels were interrupted by making single transverse cuts through (parts of) the stem with a razor blade (Zimmermann *et al.*, 1995 b and unpublished results).

Despite the numerous convincing model and plant experiments, it has been argued that high tensions would be eliminated upon piercing of the vessel by the capillary tip (Milburn, 1996). Subsequent insertion of two probes into the same vessel of a root of a transpiring maize plant showed that this is obviously not the case (Figure 3).

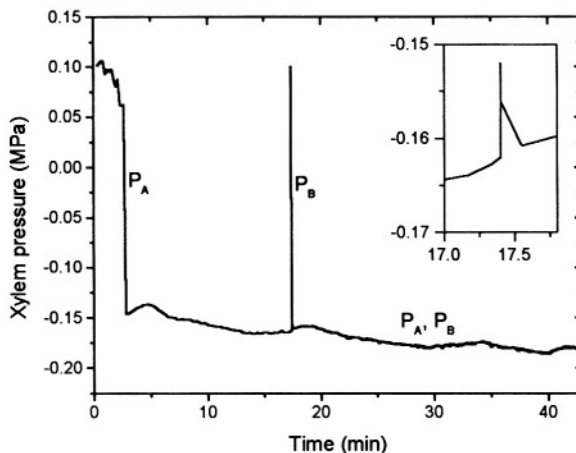


Figure 3. Xylem pressure measurement in an intact maize root (bathed in nutrition medium) showing the responses to the consecutive insertion of two xylem pressure probes (P_A and P_B , respectively) into the same metaxylem vessel. The measurement was performed under laboratory conditions at a light intensity of $700 \mu\text{mol m}^{-2} \text{s}^{-1}$. Data from Schneider *et al.* (1997 a), modified. Note that the insertion of the probe P_B is indicated by a small pressure spike read by probe P_A which dissipated rapidly (see inset). Then, both probes read the same pressure values.

13.3.3 XYLEM PRESSURE-POTENTIAL PROBE

Measurements of the electrical potential difference between the solution bathing the root and the xylem, i.e. the so-called trans-root potential, and of (longitudinal) electrical gradients in the xylem conduit, provide a way to elucidate the contribution of electrical forces to long-distance salt and water transport. In all theoretical considerations (see also this article) electrical coupling is neglected. This means that salt is treated as a non-electrolyte.

The incorporation of electrical forces into the flow equation of the thermodynamics of irreversible processes is possible, but requires accurate experimental data, which were hitherto not accessible. Some authors determined the radial electrical potential difference in the root (Davis and Higinbotham, 1969; De Boer *et al.*, 1983) or hypocotyl (Okamoto *et al.*, 1978) after excision of the shoot, or measured longitudinal electrical gradients on root or shoot segments (e.g. De Boer and Prins, 1985). Other authors (Dunlop, 1982; Walker and Graham, 1987) recorded the trans-root potential of intact plants by insertion of a microelectrode into the root tissue. These approaches suffer from several shortcomings as discussed elsewhere (Wegner and Zimmermann, 1998; for a recent review, see De Boer, 1999).

The xylem pressure-potential probe (Figure 1B) does not share the disadvantages of working with excised plant organs. In contrast to measurements with conventional microelectrodes, proper positioning of the microelectrode in a xylem vessel is indicated by the pressure drop, and clogging of the microcapillary tip can easily be detected and subsequently removed by the displacement of the metal rod within the probe. The integrated microelectrode in the probe can be used to separately determine the radial resistance of the root in order to verify that insertion of the capillary does not short-circuit the tissue.

Implementation of a potential-recording electrode within a xylem pressure probe requires some modifications of the probe as shown in Figure 1B. The microcapillary of the probe is connected via a pressure-tight seal with a horizontal borehole drilled into the perspex chamber of the probe. This borehole contains the Ag/AgCl electrode. About 4 mm of the electrode tip is coated galvanically with silver alginate, whereas the remaining part is covered with a thin teflon layer. The tip of the electrode is positioned close to the pressure-tight seal between the microcapillary and the perspex chamber. Electric contact between the xylem sap and the electrode is achieved with degassed 50 mM KCl solution. The vertical borehole provides a connection to the pressure transducer and the metal rod (for injection of pressure pulses into the punctured vessel; see above). This borehole is filled with deionized and degassed water. For further details, the reader is referred to Wegner and Zimmermann (1998).

13.3.4 PRESSURE CHAMBER TECHNIQUE

The xylem pressure probe technique is a direct, minimal-invasive technique for the measurement of the equilibrium hydrostatic pressure (or tension) in a single vessel. In contrast, the pressure chamber (Scholander *et al.*, 1965; as well as related techniques such as the centrifugation and psychrometric methods; see the literature quoted in Zimmermann *et al.* (1993 b); Holbrook *et al.* (1995)) is a "gross" technique that measures the "water availability" or average values of "water potentials" across the tissue. It involves pressurizing the phase surrounding an excised leaf or twig until water is forced (at the so-called balancing pressure) through the cut end. It is assumed that the balancing pressure value is numerically equal to the tension that existed in the vessels before excision of the leaf or twig. This method which has never been calibrated or tested convincingly in model experiments yielded extremely negative values for the "xylem pressure" (-3 to -6 MPa e.g. for mangroves and other plants subjected to saline solutions; Scholander *et al.*, 1966; Scholander, 1968; Longstreth and Strain, 1977).

These values are in contradiction to the thermodynamic considerations mentioned above and to the physical behaviour of thermodynamically metastable (e.g. super-heated) fluids.

Combined pressure probe and pressure chamber measurements on excised leaves have shown (Balling and Zimmermann, 1990) that external pressure is not immediately and completely transmitted to the xylem², except in well-hydrated (non-transpiring) leaves. Physics shows that excess pressure is required not only for the lift of the water column in the excised leaves, but in particular for the compression of the gas phases in the leaf, for mass transfer through the apoplastic space in order to establish a water continuum between the xylem and the external gas phase, as well as for the compensation of the elastic restoring forces and of the osmotic pressures in the apoplastic and symplastic compartments.

The controversy surrounding the validity of the pressure chamber technique was recently settled by concurrently employing the pressure chamber and pressure probe techniques. In the study of Melcher *et al.* (1998), simultaneous measurements were made with the probe inserted into xylem vessels of intact, transpiring leaves, and with the pressure chamber in both previously transpiring and previously non-transpiring, covered leaves, that were rapidly detached prior to the pressure chamber experiment from the same plant (Figure 4A). The experiments performed on nearby leaves of maize and sugarcane (data for the latter species are not shown) clearly demonstrated that large deviations between the pressure chamber and the probe readings were only observed when balancing pressure values were obtained from transpiring leaves (Figure 4B). The discrepancy in the values measured by the two techniques increased with the progressing day, that is with increasing transpiration. In contrast, the balancing pressure values obtained on the non-transpiring (covered) leaves were similar in absolute magnitude to those pressure values determined with the probe on nearby leaves, regardless of whether they were transpiring or not.

Provided that the pressure chamber measured xylem pressure, the differences in the balancing pressure values of transpiring and non-transpiring leaves would contradict physics stating that the xylem pressure should be equal in covered and non-covered leaves on the same plant (as can also be demonstrated by means of the xylem pressure probe; unpublished results) because of the very low resistances in the xylem conduit. Thus, the conclusion can be drawn that a large part of the pressure built up in the pressure chamber for extrusion of water through the cut end is due to the (transpiration-dependent) presence of gas in the leaf and, in turn, to the related changes in the water continuity and osmotic profiles within the leaf.

Indeed, NMR imaging of the petiole of pressurized leaves (Zimmermann *et al.*, 2000; Wagner *et al.*, unpublished data) favoured the observations of Melcher *et al.* (1998). The NMR experiments showed that spin density (i.e. water concentration) did not significantly change during and after pressure application of up to 0.5 MPa. However, **T₁-weighted** images showed a significant (and reversible) increase in signal intensity when increasing pressures were applied. Since other effects, such as changes in the **O₂-concentration**, could be excluded, these findings could be only explained by a shift of

² Note that pressure application to the root generally resulted in a 1 : 1 (or only slightly less) response in xylem pressure (Zimmermann *et al.*, 1991; Benkert, 1994).

water within the leaf petiole from large compartments into small ones, e.g. from large (turgescient) cells to small, less turgescient ones or to air-filled intercellular spaces. This finding supports the above statement that a large part of the external pressure is not used for axial lifting of the water column in the vessels.

Thus, the pressure chamber can only be used for the measurement of xylem pressure provided that the plants are well hydrated and non-transpiring and that their xylem osmotic pressure is negligible (which is not necessarily the case for mangroves and other halophytes). Therefore, the authors of this review article agree with Canny (1993) and others in the field that the pressure chamber (and the other indirect techniques; for critical analyses see e.g. Zimmermann *et al.*, 1993b; Shackel, 1996) should be used neither for measurements of xylem pressure nor for the determination of xylem sap composition (via analysis of sap squeezed out of the cut end of the plant organ).

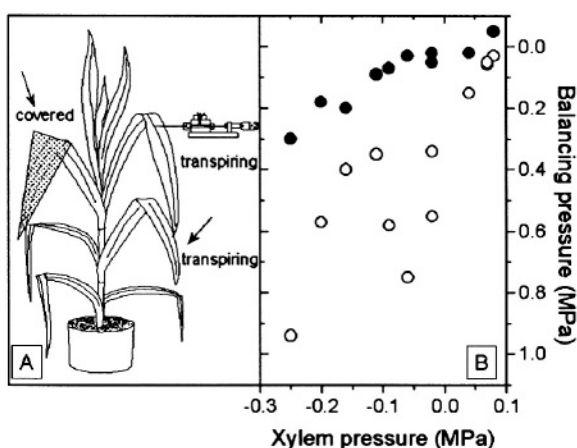


Figure 4. Schematic diagram of the set-up (A) used for the determination of the relationship (B) between probe measurements of xylem pressure in transpiring leaves and balancing pressures measured with the pressure chamber on transpiring leaves (open symbols) and covered (i.e. non-transpiring; indicated by the grey triangle in A) leaves (filled symbols) of maize. The measurements were performed on nearby leaves (see A) of 1.5- to 2-m-tall plants in a tropical greenhouse. The leaves used for pressure chamber measurements are marked with arrows. The data were taken from Melcher *et al.* (1998), redrawn and modified. For explanation of the results, see text.

13.4 Xylem and cell turgor pressure and their dependence on light and humidity

13.4.1 LIGHT/HUMIDITY EFFECTS

Xylem and cell turgor pressure in higher plants generally respond (but not always; see Schneider *et al.*, 1999) in a characteristic manner to changes in light intensity, temperature and/or relative humidity, independently of the growth conditions.

Typical examples for changes of the xylem pressure upon changes in illumination are shown in Figure 5. Insertion of the probe into a single stem vessel of a potato plant at a relatively low light intensity yielded a stable slightly negative pressure of -0.06 MPa within a few seconds. A sudden increase of the light intensity by using an additional light source (see dashed line in Figure 5) did not lead to an immediate response in the xylem pressure. For about 4–5 min the pressure remained constant. Then the tension progressively increased with time. After about 15 min when the pressure had reached a value of -0.14 MPa, the light was switched off. The xylem pressure again responded with a delay of several minutes and subsequently increased very slowly, only after about 1 h returning to its original value.

This delayed response was only observed when the illumination phase was too short for establishing the equilibrium value. When the light phase was extended to at least 1 h (see second to fourth run in Figure 5) it can be seen that upon illumination an “overshoot reaction” occurred and that the final equilibrium pressure was reached only after about 1–2 h (this time period varied with plant species; compare Wegner and Zimmermann, 1998; Schneider *et al.*, 1999). There was evidence that the “overshoot reaction” resulted from changes in transpiration and/or from water exchange with the surrounding tissue (see Eq. 3). Provided that the equilibrium value was established, switching off the light resulted in a very fast re-establishment of the original pressure in the dark. This light-dependent xylem pressure pattern could be repeated several times on the same plant (Figure 5).

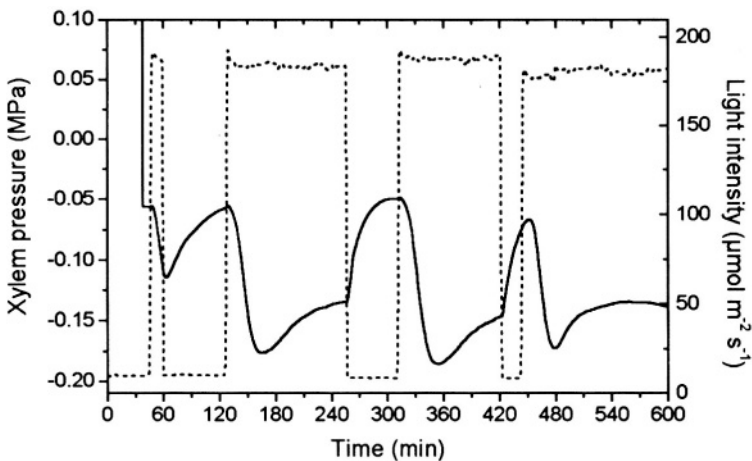


Figure 5. Xylem pressure response (solid line) upon repeated changes in light intensity (dashed line) measured in the stem of an intact soil culture potato plant under laboratory conditions (temperature 23°C to 27°C; relative humidity 23% to 28%). The vessel was probed at a light intensity of $10 \mu\text{mol m}^{-2} \text{s}^{-1}$, indicated by a rapid drop of the pressure from (above-) atmospheric values to a stable negative value of -0.06 MPa. For further details, see text.

Studies on wheat and maize using the xylem pressure-potential probe revealed that the light-induced xylem pressure changes were also accompanied by changes in the trans-root potential (see Figure 7C and Wegner and Zimmermann, 1998). Interestingly, the

electrical response of the root upon illumination was much faster than the xylem pressure response (Wegner and Zimmermann, 1998). Even though the underlying mechanisms are not fully understood, this finding suggests that electrical effects on long-distance water and salt transport must be taken into account and, furthermore, indicates the existence of longitudinal electrical gradients within the vessels. Preliminary experiments on detached leafy shoots supported this assumption.

In contrast to light exposure, changes in the relative humidity always resulted in an immediate response of the xylem pressure. Figure 6 illustrates a typical experiment performed on a tomato plant in a climate chamber. After probing of a vessel at a low relative humidity, the plant was exposed to short- and long-term changes in the relative humidity. It is evident that the xylem pressure almost immediately responded to the changes in the relative humidity (except when the leaves were covered with water; see Figure 6, horizontal arrow), i.e. it increased with increasing relative humidity and decreased with decreasing relative humidity.

Changes in xylem pressure upon illumination or changes in relative humidity were always immediately reflected by corresponding changes of the cell turgor of the tissue cells (Thürmer *et al.*, 1999 and unpublished results), indicating a tight hydraulic coupling between the xylem and tissue cell compartments (as predicted by Eq. 3).

Consistent with this, the xylem pressure remained unaltered when the upper and lower surfaces of the leaves were completely covered with low viscosity oil, thus preventing water loss from the plant (Balling and Zimmermann, 1990). Under these conditions, the

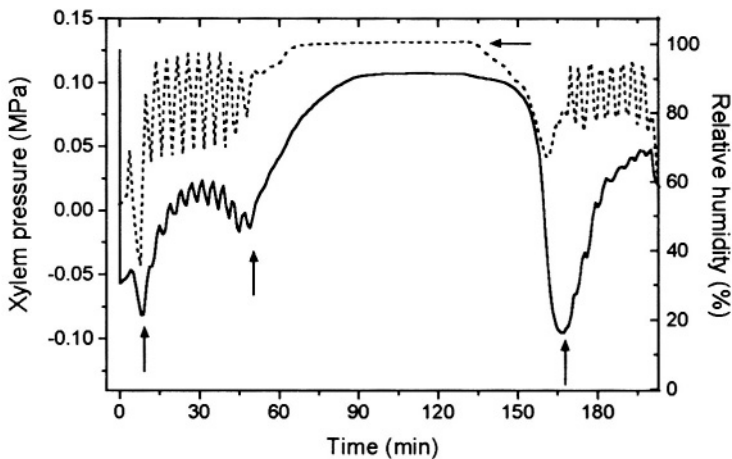


Figure 6. Xylem pressure response (solid line) of a soil culture tomato plant to changes in relative humidity (dashed line). Measurements were performed in a climate chamber (temperature 23°C - 25°C) at a constant light intensity of $10 \mu\text{mol m}^{-2} \text{s}^{-1}$. The relative humidity was increased by temporarily blowing humidified air into the climate chamber. It is evident from the data that the xylem pressure changed immediately in response to an increase in relative humidity (upwardly directed arrows) and exceeded the atmospheric value at a relative humidity of about 100%. This was accompanied by guttation. After some time the leaves were covered completely with water, which subsequently prevented an immediate xylem pressure response upon lowering of the relative humidity (horizontal arrow). For further explanations, see text.

cell turgor pressure did also not change, again supporting the view that the magnitude of the xylem pressure is exclusively determined by the “water potential” of the tissue cells (as predicted by Eq. 3) and that no changes in the xylem pressure are to be expected if the cell turgor pressure remains constant.

Experiments performed on the roots and stems of various plant species showed that the changes in xylem pressure and in turgor pressure induced by transpiration variations were not always equal (Schneider *et al.*, 1997 b, 1999). Sometimes the ratio between these two parameters was only 1 : 0.3 (e.g. for maize roots) to 1 : 0.5 (for wheat roots, potato stems etc.). According to Eq. 3 this would suggest that the drop in turgor pressure was accompanied with some cell volume shrinkage, leading to a corresponding increase of the internal osmotic pressure (this was, e.g., observed for a drought-resistant resurrection plant; Schneider *et al.*, 1999). An alternative and very likely explanation is that the generation of turgor and osmotic pressure gradients upon illumination (Rygol *et al.*, 1993) affects turgor pressure changes considerably, depending on the insertion depth of the turgor pressure probe microcapillary. However, due to technical difficulties cells directly adjacent to the xylem were not measurable, especially in the case of very thick tissues consisting of a great number of cell layers.

Preliminary measurements of the average cellular osmotic pressure in various plants allowed (together with the simultaneous determination of the xylem and cell turgor pressure) the estimation of the osmotic pressure in the xylem according to Eq. 3. This yielded rather high values for this parameter (about 0.3 - 0.4 MPa; Thürmer *et al.*, 1999; Schneider *et al.*, 1999).

13.4.2 LIGHT-INDUCED OSCILLATIONS OF THE DRIVING FORCES

Under conditions of increased light intensity (for wheat also often at low illumination; see Wegner and Zimmermann, 1998), periodic forward and backward regulations in xylem pressure occurred for various plant species which often covered a pressure range of more than 0.2 MPa (Schneider *et al.*, 1997b; Wegner and Zimmermann, 1998). Periodic changes in the transpiration rate (i.e. in stomatal aperture; compare Farquhar and Cowan, 1974; Raschke, 1975) were linked to these xylem pressure “oscillations”, with highest transpiration corresponding to lowest xylem pressure values and vice versa (Figure 7A). However, the transpirational changes were always slightly (by about 1 min) ahead of the pressure changes. The pressure oscillations disappeared reversibly when the plant leaves were submerged in water (Schneider *et al.*, 1997 b). Xylem pressure and cell turgor pressure measurements (which were partly performed simultaneously; Schneider *et al.*, 1997 b) and simultaneous xylem pressure/trans-root potential measurements, respectively, showed for intact roots of transpiring hydroculture wheat plants that the cell turgor pressure of adjacent tissue cells (Figure 7B) as well as the trans-root electrical potential (Figure 7C) showed similar oscillations (see also Schneider *et al.*, 1997 b; Wegner and Zimmermann, 1998). Interestingly, the membrane potential of the outermost cortical cells remained unaffected by the transpirational oscillations (data not shown; see Wegner and Zimmermann, 1998).

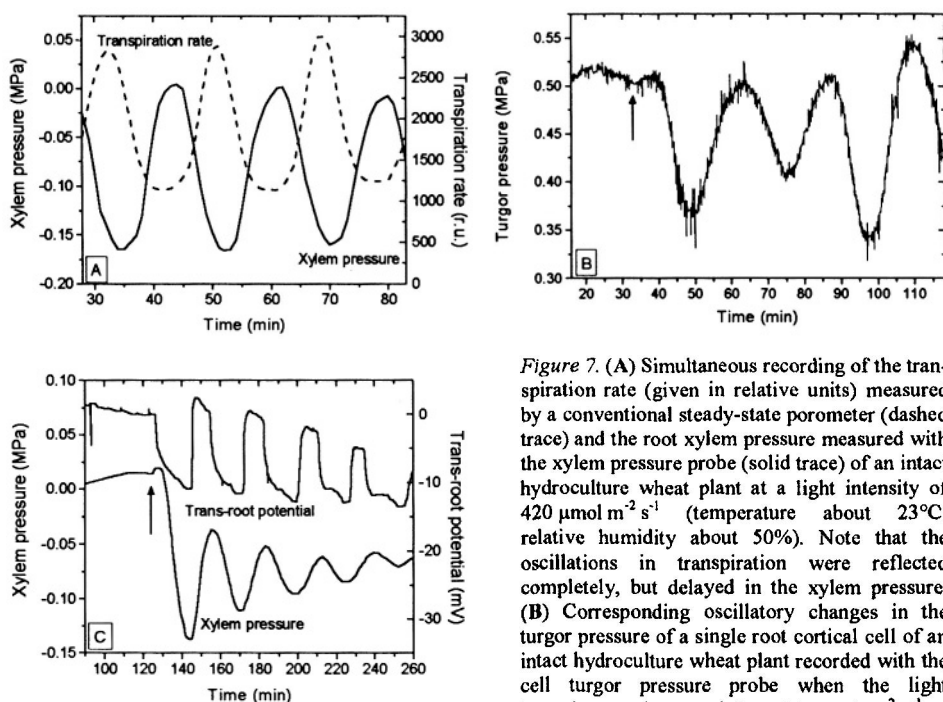


Figure 7. (A) Simultaneous recording of the transpiration rate (given in relative units) measured by a conventional steady-state porometer (dashed trace) and the root xylem pressure measured with the xylem pressure probe (solid trace) of an intact hydroculture wheat plant at a light intensity of $420 \mu\text{mol m}^{-2} \text{s}^{-1}$ (temperature about 23°C ; relative humidity about 50%). Note that the oscillations in transpiration were reflected completely, but delayed in the xylem pressure. (B) Corresponding oscillatory changes in the turgor pressure of a single root cortical cell of an intact hydroculture wheat plant recorded with the cell turgor pressure probe when the light intensity was increased from $10 \mu\text{mol m}^{-2} \text{s}^{-1}$ to about $300 \mu\text{mol m}^{-2} \text{s}^{-1}$ (arrow). (C) Simul-

taneous recording of the oscillations of the xylem pressure (lower trace) and the trans-root potential (upper trace) of an intact hydroculture wheat plant by using the xylem pressure-potential probe (temperature 24°C ; relative humidity 28%). The root was bathed in standard medium as described in Wegner and Zimmermann (1998). Oscillations were induced by an increase in light intensity from $10 \mu\text{mol m}^{-2} \text{s}^{-1}$ to $350 \mu\text{mol m}^{-2} \text{s}^{-1}$ as denoted by the arrow.

13.5 Xylem and cell turgor pressure responses of roots of transpiring plants to salt treatment

13.5.1 THEORETICAL CONSIDERATIONS

The xylem pressure probe can also be used to directly measure the pressure change in the root xylem induced by the addition of salt (i.e. by a change in the osmotic pressure of the bathing solution). The osmotically induced changes in xylem pressure can be analyzed in terms of the thermodynamics of irreversible processes if the root tissue is treated as a two-compartment system, that means that the aqueous xylem phase and the solution bathing the roots are separated by a single transport barrier with semipermeable properties.

For osmotic equilibrium, where the net water flow, J_v , through the root is zero, the following relationship applies for changes in the hydrostatic pressure of the xylem, ΔP_x , upon a change in the external osmotic pressure, $\Delta \pi_e$,

$$\Delta P_x = \sigma_r \Delta \pi_e \quad (4)$$

where σ_r is termed the radial reflection coefficient of the root tissue. The value of σ_r depends on the properties of both the transport barrier and the solute. In a system containing more than one solute, each solute will generally have a different value of the radial reflection coefficient.

If the root of a plant behaves as a perfect osmometer (or more accurately as a Hepp-type osmometer; see above), σ_r assumes a value of unity. This implies that a change in the osmotic pressure of the external medium (calculated from the concentration, c , according to van't Hoff's law $\pi = cRT$) results in an equivalent change of xylem pressure. On the other hand, σ_r smaller than unity means that only a correspondingly low fraction of the osmotic pressure becomes effective. In the extreme case of $\sigma_r = 0$, the solutes do not exert any osmotic pressure on the tissue (so that the change in xylem pressure is zero).

The radial reflection coefficient, describing the coupling between solute and water flow (in the absence of any electrical gradients), is given by (Zimmermann and Steudle, 1978):

$$\sigma_r = -L_{PD} / L_P \quad (5)$$

where L_{PD} is the coupling coefficient between the solute and water flow within the transport barrier (= the coefficient of osmotic flow) and L_P the hydraulic conductivity of the barrier.

It is important to note that σ_r does not describe the solute permeability of the transport barrier. Thus, a low reflection coefficient does not necessarily mean that the permeability coefficients of the solutes are very high (Anderson and Malone, 1974; Hill, 1982, 1994). Changes in σ_r can be induced by changes in L_{PD} , by changes in L_P or by changes of both parameters.

There is also the possibility of σ_r being negative. This would mean that the transfer of the solute occurs more rapidly than that of the solvent, once the osmotic pressure was changed in the external solution. Such cases are known for salt transport and are called negative anomalous osmosis; they are of special interest in the case of electrolytes passing through alternatively charged membranes, e.g. through so-called mosaic membranes (literature quoted in Zimmermann and Steudle, 1978).

Cases are also conceivable where σ_r assumes values higher than unity, that means that a change in the external osmotic pressure results in an amplified change of xylem pressure. The significance of $\sigma_r > 1$ can easily be understood when the transport equation for a solute is considered at $\pi = 0$. Under these circumstances (if we neglect electrical coupling), the solute flow, J_s ($\text{mol cm}^{-2} \text{s}^{-1}$), is only driven by the "solvent

drag" effect (Zimmermann and Steudle, 1978) induced by the water flow, J_v ($\text{cm}^3 \text{cm}^{-2} \text{s}^{-1}$), and is given by Eq. 6:

$$J_s = (1 - \sigma_r) c J_v \quad (6)$$

where c is the mean concentration between the xylem and the medium compartments.

It is clear from Eq. 6 that in the case of $\sigma_r > 1$, J_s must be opposite to J_v . Such a condition can be realized when the symplastic water pathway through the root tissue is inhibited (see below).

If the radial reflection coefficient is experimentally determined by measurements of the osmotically induced changes in xylem pressure (see Eq. 4) one has to take into account that the condition of $J_v = 0$ can only be established in excised roots or intact, non-transpiring plants. For an intact, transpiring plant Eq. 4 may nonetheless be applied if σ_r is used as an operational parameter. In this case, the root represents a "water transport device" (operating under steady state conditions) and, thus, the radial reflection coefficients for the solutes may strongly depend on the magnitude of the transpiration-dependent water flow through the root tissue (see below).

13.5.2 SHORT-TERM SALT TREATMENT AND XYLEM PRESSURE RESPONSE

Figure 8 illustrates typical pressure profiles recorded after puncturing of a conducting xylem vessel in the root of an intact, transpiring maize (A) and wheat plant (B), respectively, under laboratory conditions at a low light intensity. It is obvious that the absolute pressures in the root xylem of maize and wheat were in the sub-atmospheric pressure range when the roots were bathed in nutrition medium (osmotic pressure about

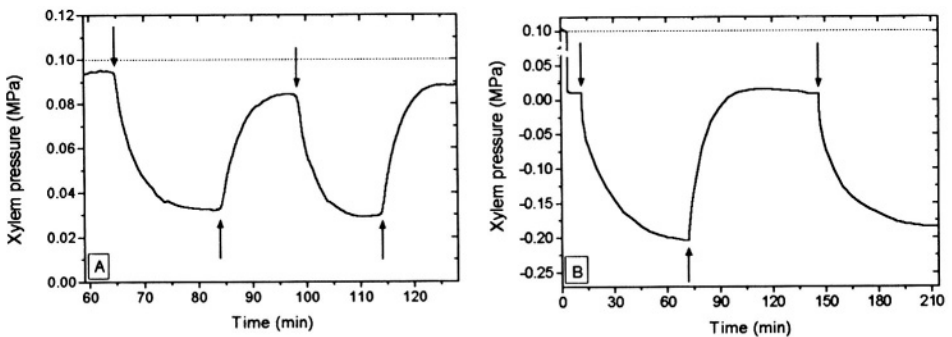


Figure 8. Typical responses of the root xylem pressure of an intact maize (A) and wheat plant (B) upon addition of 50 mM NaCl ($\Delta\pi = 0.23$ MPa). The measurements were performed under laboratory conditions (light intensity about $10 \mu\text{mol m}^{-2} \text{s}^{-1}$; temperature 22°C ; relative humidity 45%). Downwardly directed arrows = replacement of the nutrition medium by the saline solution; upwardly directed arrows = replacement of the saline solution by nutrition medium. The σ_r -values extracted from the curves yielded *ca* 0.3 for maize and *ca* 0.9 for wheat. The dotted line indicates atmospheric pressure. Note that only parts of the experiments are shown.

0.03 MPa). When the roots were subjected to a sudden short-term NaCl stress, the xylem pressure immediately decreased to (more) negative values within a few minutes. Although the response time of the roots to osmotic changes was comparable to that reported for excised roots in the literature (Azaizeh and Steudle, 1991; Steudle, 1993), the drop in xylem pressure could generally not be fitted by a single exponential curve. In addition, the root xylem pressure of salt-treated intact plants did not always remain constant.

Lowering of the xylem pressure by salt treatment was often followed by forward and backward changes in xylem pressure including short-term pressure fluctuations (data not shown; see Zhu *et al.*, 1995; Schneider *et al.*, 1997 b). Similar alterations in the xylem pressure were observed after replacing the salt solution by a nutrition medium. These “regulation phenomena” again seemed to be closely correlated with changes in stomatal conductance, but were apparently also linked with the shift of water between the xylem and tissue compartments.

Simultaneous probing of a xylem vessel and a cortical cell in the roots of glycophytes revealed that changes in xylem pressure induced by salt stress were clearly reflected in turgor pressure changes, as already found for illumination (see above; Schneider *et al.*, 1997b). This again supported the view of a very close hydraulic connection between the tissue cells and the xylem as predicted by Eq. 3.

A very interesting result, extracted from experiments in which glycophytes were treated with saline solutions under laboratory conditions (Figure 8), was that the xylem pressure response of maize to the salt treatment was considerably attenuated, i.e. less than expected from the osmotically effective concentration of the salt (Figure 8A), when low light intensities were chosen (Zhu *et al.*, 1995; Schneider *et al.*, 1997 b). This was not the case for wheat (see Figure 8B) and barley (Schneider *et al.*, 1997 b). This finding

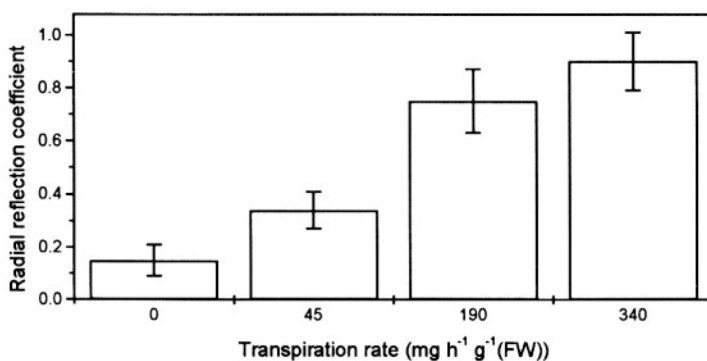


Figure 9. Values of the radial reflection coefficient (σ_r) for NaCl measured on intact maize roots under different transpiration conditions. The σ_r -values were determined by measurements of the xylem pressure response upon addition of 25 mM NaCl (or 50 mM NaCl; first column) to the external medium. The data represent average values of at least 6 independent experiments. The standard deviations are indicated by the bars. FW = fresh weight of the leaves.

suggested that the radial reflection coefficient for NaCl (and other salts) was substantially less than unity in the case of maize plants, in agreement with the results obtained for excised roots (Steudle, 1989).

However, a closer investigation of the pressure response of intact plants to short-term salt stress revealed that the magnitude of σ_r depended on the (species-dependent) transpiration rate. A stepwise increase in the transpiration rate (e.g. by an increase in light intensity) resulted in a corresponding increase of the σ_r -values of maize up to unity (Schneider *et al.*, 1997 b; see Figures 9 and 11). Consistent with this, measurements in a tropical greenhouse on maize showed (Figure 10) that diurnal changes in the transpiration rate were completely reflected in corresponding changes of the radial reflection coefficient (Schneider *et al.*, 1997 a). The cellular reflection coefficients of glycophytes for salt showed a somewhat similar trend with transpiration, but the effect of the transpiration rate on this parameter was less than on σ_r . The mechanism underlying the transpiration-dependent (species-specific) pattern of the radial and cellular reflection coefficients of the roots of glycophytes (and presumably of halophytes) is not yet clear, but may result from (flow-dependent) concentration-polarization and sweep-away effects in the roots of intact plants.

From the above data we can conclude that in non-transpiring plants (i.e. during darkness and at high relative humidity) the xylem pressure response was close to zero upon salt treatment, whereas in highly transpiring plants at noon time the response was 1 : 1. In other words, non-transpiring plants are apparently less sensitive to short-term salt exposure than transpiring plants. Halophytes also seem to share this feature of glycophytes. For example, xylem probe measurements in the roots of the mangrove *Rhizophora mangle* have shown (Zimmermann *et al.*, 1994 b) that changes of the

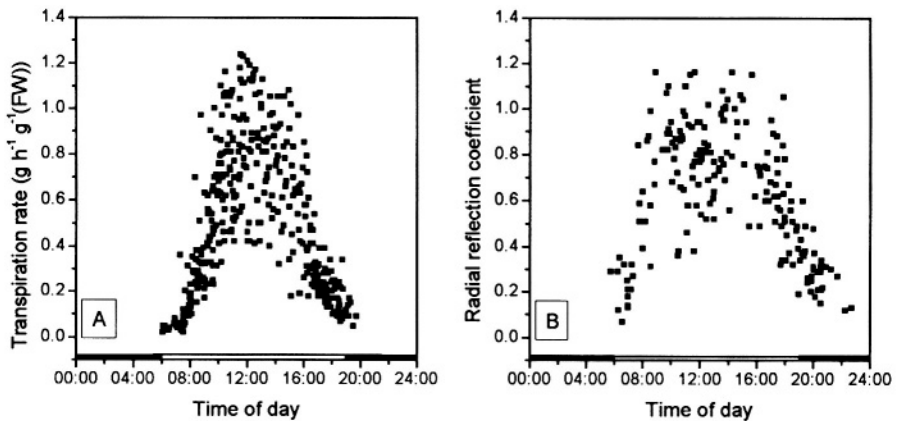


Figure 10. Diurnal changes of the transpiration rate (A) and the radial reflection coefficient (σ_r) for NaCl (B) of roots of intact maize plants measured in a tropical greenhouse. The σ_r -values were determined by measurements of the xylem pressure response upon addition of NaCl (up to 100 mM) to the external medium. FW = fresh weight of the leaves. The data were taken from Schneider *et al.* (1997 a), redrawn and modified. Note that the changes in the σ_r -values closely followed the diurnal changes in the transpiration rate (which was quite variable because of large and rapid changes in the weather conditions).

osmolality of the external sea water were not reflected in corresponding changes of the xylem pressure (i.e. σ_r was close to zero). This result could retrospectively be explained by the extremely low transpiration flow determined for this plant (Zimmermann *et al.*, 1994b). This finding has implications for the water ascent in this species: the development of high tensions in the xylem is not expected and water lifting must be achieved by alternative driving forces (Zimmermann *et al.*, 1994 a, b). Very low xylem tensions (of about 0.1 MPa) are in agreement with results obtained by Scholander *et al.* (1962) on mangroves by the leafy twig/vacuum method introduced by Renner (1925) and with observations using the xylem pressure probe (Zimmermann *et al.*, 1994b), but contradict the pressure chamber data (Scholander *et al.*, 1966; Scholander, 1968).

13.5.3 AMPLIFICATION OF SALT STRESS EFFECTS ON XYLEM PRESSURE IN Hg-TREATED ROOTS OF MAIZE

Even though Hg could have various effects on cellular functions and metabolism there is evidence that this heavy metal ion blocks water channels of the cellular pathway in the roots (Maggio and Joly, 1995), thus increasing the hydraulic resistance for xylem-bound water flow. Introduction of artifactual resistances into the xylem-bound water flow (e.g. by transverse cuts or by reduction of the expression rate of water channels in the roots; Kaldenhoff *et al.*, 1998) usually resulted only in a temporary increase of the xylem tension associated with a decrease in the xylem flow. Plants have the ability to overcome such resistances within a few days, e.g. in the case of transverse cuts by flow through the tissue or in the case of a reduction of water channels by enlargement of the root surface (i.e. root growth; Kaldenhoff *et al.*, 1998).

Information on long-term effects of Hg on xylem flow under saline conditions is not available at present. However, short-term experiments have yielded very interesting results. Addition of 10 μM HgCl_2 to the nutrition medium prior to salt exposure, always

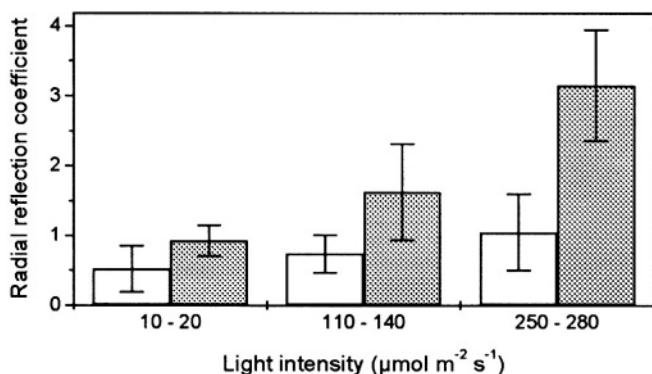


Figure 11. The dependence of the radial reflection coefficient (σ_r) of roots of intact maize plants on NaCl at different light intensities measured in the absence (white columns) and in the presence of 10 μM HgCl_2 (grey columns). The σ_r -values were determined by measurements of the xylem pressure response upon addition of 25 mM NaCl to the external medium. The data represent average values of at least 3 independent experiments. The standard deviations are indicated by the bars.

resulted in a continuous drop of the xylem pressure. A new water equilibrium was reached after quite variable times ranging from minutes to several hours. Parallel measurements of the transpiration rate gave no clear-cut indication that this parameter was affected by the Hg-treatment. Subsequent replacement of the Hg-containing nutrition medium by a solution additionally containing 25 mM NaCl caused a drop in xylem pressure which was substantially larger than expected from the change in the osmotic pressure of the medium. In Figure 11, the corresponding radial reflection coefficients, extracted from the final xylem pressure values after short-term salt treatment at various light intensities for control plants as well as for Hg-treated plants are given. It is evident that in the presence of Hg the response of the xylem pressure to salt treatment could be amplified by a factor of up to 3 at increased light intensities. Such high σ_r -values are consistent with the above theoretical considerations (Eq. 6) if we assume that the symplastic pathway for radial water flow towards the xylem is inhibited by Hg (i.e. L_P is very low compared to L_{PD} , see Eq. 5), but that the net radial solute flow is still functional and inwardly directed.

13.6 Perspectives

Probe measurements obviously provide a powerful tool for the evaluation of water relations of the xylem conduit and of the hydraulic and electrical coupling to the surrounding tissue under saline and other environmental conditions. Substantial progress can be expected in the nearest future, when probes with integrated ion-selective microelectrodes will be available which will allow “on-line” monitoring of the transport of ions (such as K^+ , Na^+ , Cl^-) on the level of single xylem vessels in intact plants in addition to the pressure and potential gradients. This and the combination of the various probe techniques with the large number of (non-invasive) NMR-imaging techniques (developed in recent years for separately measuring xylem and phloem flow; see Rokitta *et al.*, 1999 a, b) as well as Na^+ -transport through root tissues will certainly lead to a more consistent understanding of the complex coupling pattern of water and solute transport against gravity in higher plants.

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CHAPTER 13

SALINITY, GROWTH AND PHYTOHORMONES

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Abstract

Salinity reduces the ability of plants to take up water, and this quickly causes reductions in the growth rate, along with a suite of effects identical to those caused by water stress. Later, there may be a salt-specific and additional effect on growth: if excessive amounts of salt enter the plant they will eventually rise to toxic levels in the older transpiring leaves, and reduce the photosynthetic capacity of the plant. Salinity can therefore affect growth via changed water relations, hormonal balance, or carbon supply, the relative importance of each process depending on the time scale of the response.

13.1 Introduction

Great diversity exists between species in ability to grow and reproduce in saline soil. Contributing to this diversity are differences between species in ability to control salt uptake from the soil, and to compartmentalize it effectively at the cellular level. Yet even in the most salt-tolerant species, growth rates are greatly reduced by salinity. How this comes about is the subject of this chapter.

The approach taken here is first to illustrate the differences that occur between species in their responses to salinity, and to divide these into osmotic and salt-specific parts. The underlying concepts to emerge are that growth-limiting factors are predominantly those induced by osmotic stress, but in species that have a high rate of salt uptake, or cannot compartmentalize salt effectively in vacuoles, salt-specific effects develop with time, impose an additional stress on the plant through failing capacity to produce photoassimilate, and give rise to the categories of 'salt-sensitive' and 'salt-tolerant'.

Next, the central topic is addressed, of the role of hormones in the regulation of growth in saline soil. The effects of salinity, and other environmental stresses, have been

summarized in an excellent review by Naqvi (1994), who gives a comprehensive overview of hormonal relations and interactions, and their role in the alleviation of stress. Most of the present chapter forms a discussion of *whether* hormones provide the main regulation, rather than *which* hormones are acting. Whether water status, hormonal regulation or supply of photosynthate exerts the dominant control over growth is an issue hotly debated for the last two decades, and not yet resolved. More work has been done on this topic for plants in dry soil rather than in saline soil, and these results are drawn on.

To understand whether or not hormones are the major factors controlling growth rates, this chapter attempts to define the conditions in which they might dominate the growth response, in contrast to those in which water relations or carbohydrate supply might dominate. These multiple controls are unraveled by tracking the projectory of the growth response over time. This is done within a time frame extending from seconds to months. Over this time span, effects of salinity move from reductions in rates of cell expansion, cell production, and leaf emergence, to developmental changes involving the production of lateral primordia production and the formation of reproductive organs.

13.2 Plant diversity in growth responses to salinity

Salt tolerance is usually assessed by physiologists as the percent biomass production in saline versus control conditions over a prolonged period of time. Dramatic differences are found between plant species (Chapter 3). As illustrated in a review by Greenway and Munns (1980), after some time in 200 mM NaCl, a salt-tolerant species such as sugarbeet might have a reduction of only 20% in dry weight, a moderately tolerant species such as cotton might have a 60% reduction, and a sensitive species such as soybean might be dead. On the other hand, a halophyte such as *Suaeda maritima* might be growing at its optimum rate (Flowers *et al.*, 1986).

A complete survey of the salt tolerance of crops, vegetables and fruit trees has been published by Maas and Hoffman (1977), and updated by Francois and Maas (1994). These data are for yield, not vegetative growth. They show for each species a threshold salinity below which there is no reduction in yield, and then a regression for the reduction in yield with increasing salinity. However, with young plants a threshold is rarely seen; with plants exposed to salinity at an early stage of seedling development there are linear reductions in both leaf area expansion and total plant biomass with increasing salinity. There are several possible reasons for a threshold salinity level for yield but not vegetative growth, the most likely one being that salinity may affect the development of reproductive structures, or the retranslocation of C and N reserves from leaves and stems to the developing fruits or seeds. The influence of salinity on reproductive development is discussed towards the end of this chapter.

Survival is sometimes used as an index of salt tolerance, when dealing with large numbers of genotypes, or when no control (non-saline) treatment can be given. Survival can be measured as the percent of plants alive after a given period of time at a

given salinity. Alternatively, if there is a range of salinities, survival can be measured as the salinity at which 50% of the plants have died. The drawback of this index is that it gives little idea of how well a plant can actually *grow* in saline conditions.

The emphasis of this chapter is on the effects of salinity on vegetative growth, as most physiological and molecular studies are done with young plants still in the vegetative stage. The term 'salt tolerance' is used in this chapter to mean the growth (dry matter production) of a plant in saline relative to control conditions.

13.2.1 THE INFLUENCE OF TIME

It is surprisingly difficult to define or quantify differences in salt tolerance between species, as the growth reduction depends so much on the period of time over which the plants have grown in saline conditions.

During a short period of time in salinity, there may be no differences in growth rate between species that have quite different reputations for salt tolerance. For example, durum wheat is much more salt-sensitive than bread wheat, due to its poor ability to exclude Na^+ , and biomass and yield are much more affected (Francois *et al.*, 1986).

Yet, over short periods of time in salinity, we have found no differences between durum and bread wheat cultivars, or between barley and triticale cultivars (Munns *et al.*, 1995).

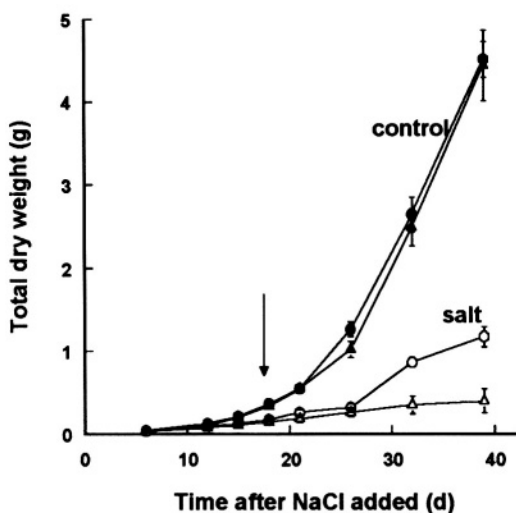


Figure 1. Two accessions of the diploid wheat progenitor *Triticum tauschii* in control solution (closed symbols) and in 150 mM NaCl with supplemental Ca^{2+} (open symbols). Circles denote the tolerant accession, triangles the sensitive one. The arrow marks the time at which symptoms of salt injury could be seen on the sensitive accession; at that time the proportion of dead leaves was 10% for the sensitive and 1% for the tolerant accession (Munns *et al.*, 1995). A similar result is given in Fortmeier and Schubert (1995).

There were no significant differences between the leaf elongation rate in the first ten days of salinization of any cultivar, including that of the one that ultimately turned out to be the most sensitive (a durum wheat) and the one (a barley) that turned out to be the most tolerant (Rawson *et al.*, 1988).

These data are consistent with the concept of a two-phase growth responses to salinity (Munns, 1993). The first phase of growth reduction is quickly apparent, and is due to the salt outside the roots. It can be called a water stress or osmotic phase, for which there is surprisingly little genotypic difference. Then there is a second phase of growth reduction, which takes time to develop, and results from internal injury. It is due to salts accumulating in transpiring leaves to excessive levels, exceeding the ability of the cells to compartmentalize salts in the vacuole. Salts then rapidly build up in the cytoplasm and inhibit enzyme activity, or they build up in the cell walls and dehydrate the cell (Flowers and Yeo, 1986; see also Chapter 8).

The two-phase growth response has been shown clearly for maize and wheat cultivars. Two maize cultivars with two-fold differences in rates of Na^+ accumulation in leaves had the same growth reduction for 15 days in 80 mM NaCl (Cramer *et al.*, 1994). Further, another two maize cultivars, again with two-fold differences in Na^+ accumulation, had the same growth reduction for 4 weeks in 100 mM NaCl, and it was not until 8 weeks that a growth difference was clearly seen (Fortmeier and Schubert, 1995). Similar results were found in wheat (Munns *et al.*, 1995). Two closely-related wheat genotypes that differed in rates of Na^+ accumulation (Schachtman *et al.*, 1991) had the same growth reduction for 2 weeks in 150 mM NaCl, and it was not until after 4 weeks that a growth difference between the genotypes was clearly seen (Figure 1). However, during the first 2 weeks the more sensitive genotype had many dead leaves (Munns *et al.*, 1995). Once the number of dead leaves increased above about 20% of the total, plant growth slowed down and many individuals started to die. These data illustrate the principle that the initial growth reduction is due to the osmotic effect of the salt outside the roots, and that what distinguishes a salt-sensitive plant from a more tolerant one is the inability to prevent salt from reaching toxic levels in the transpiring leaves.

It is not possible to be prescriptive about the length of time that plants should be grown before differences between genotypes in salt tolerance can be seen. The second phase will start earlier in plants that are poor excluders of Na^+ , such as lupins or beans, and when salinities are higher. It will also start earlier when root temperatures are higher. For plants such as rice that are grown at high temperatures, 10-15 days in salinity is sufficient to generate differences in biomass between genotypes that correlate well with differences in yield (Aslam *et al.*, 1993).

13.2.2 BETWEEN-SPECIES DIVERSITY

There is great biodiversity in salt tolerance, as illustrated in Figure 2 (Chapter 3). The data shown in the figure are for plant dry weight after a period of about a month in a range of salinities. The data are from experiments in which the salinity increased after plants were established in non-saline conditions, not for experiments when salt was

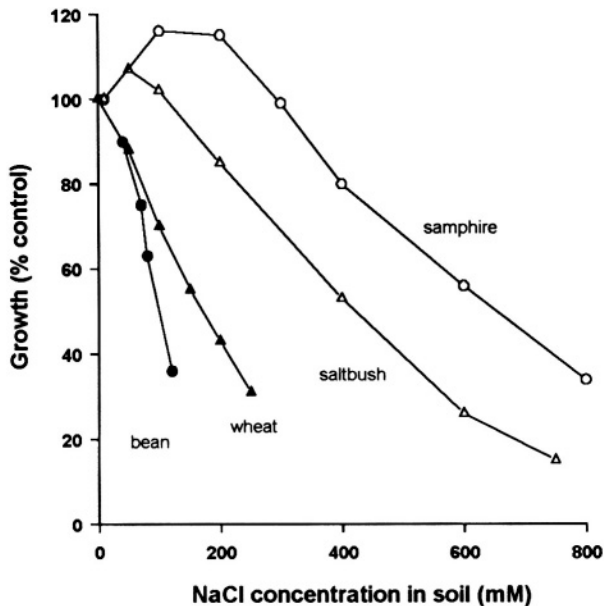


Figure 2. Biomass production of plants grown in saline soil for at least one month. Data for bean (*Phaseolus vulgaris*) are from Zaiter and Mahfouz (1993), wheat (*Triticum aestivum*) from Rawson *et al.* (1988), the saltbush *Atriplex amnicola* from Aslam *et al.* (1986), and the samphire *Halosarcia pergranulata* from Short and Colmer (1999). For the halophytes, the control is 10 mM NaCl. For wheat, supplemental Ca^{2+} was supplied so the ratio of Na : Ca was 15:1. The data for bean are for 1:1 NaCl : CaCl_2 , so for that curve the x-axis denotes Cl^- concentration.

added at germination, as these usually show a greater inhibition (e.g. Maas and Poss, 1989). Halophytes are just as sensitive as glycophytes at the germination stage.

The effect of salinity on wheat (*Triticum aestivum*) is typical of most crop species (Figure 2). Although a conception is widespread that wheat is less tolerant than barley, when Rawson *et al.* (1988) compared a number of bread wheat and barley cultivars, they found little difference in salt tolerance between those species over the period of a month. However, with extended time, durum wheat (*Triticum turgidum* ssp. *durum*) proved to be more sensitive, probably due to its poor ability to exclude Na^+ , and it died at salinities above 175 mM NaCl (Rawson *et al.*, 1988; Munns *et al.*, 1995).

The common bean (*Phaseolus vulgaris*) is one of the most salt-sensitive species (Maas and Hoffman, 1977). The supply of additional Ca^{2+} is crucial for the salt tolerance of beans, as demonstrated by Lahaye and Epstein (1971). For plants in 50 mM NaCl, the addition of 3 mM Ca^{2+} was necessary to reduce Na^+ uptake and promote growth (Lahaye and Epstein, 1971). Later studies (e.g. Zaiter and Mahfouz, 1993) showed that beans can grow quite well in 100 mM NaCl as long as Ca^{2+} is at least 5 mM. For example with a 1:1 mixture of NaCl and CaCl_2 at an EC of 10 dS m^{-1} , growth after 38 d was about 50% of controls (Figure 2).

Rice (*Oryza sativa*) is regarded as one of the more salt-sensitive crops, which is certainly true when grain yield is considered (Maas and Hoffman, 1977; Khatun *et al.*, 1995). However, growth of rice at the seedling stage can be surprisingly tolerant of salinity, at least when adequate Ca^{2+} is supplied. If this condition is met, the effect of salinity on the growth of the more tolerant rice cultivars may be no different from that of wheat shown in Figure 2. For example, growth for 42 d at 100 mM NaCl was 76% of controls when 5 mM Ca^{2+} was supplied (Muhammed *et al.*, 1987). Yet there are marked differences between genotypes, as discussed below, so the growth response of rice falls between that of wheat and bean, depending on cultivar.

Most halophytes need at least 1 mM NaCl to grow well, many need 10-50 mM NaCl to reach maximum growth, and a few succulent dicotyledonous species grow best around 200 mM NaCl (Flowers *et al.*, 1986). The adaptations of halophytes include those of glycophytes, namely salt exclusion by roots and compartmentalization of salt in vacuoles, but many species also have salt glands or bladders that excrete salt from leaves (Chapter 3). Thus even the small amount of salt arriving in leaves is excreted and leaves can remain alive for months or even years with little salt accumulation over time. Two dicotyledonous species are shown in Figure 2. Not shown is a growth response curve for a monocotyledonous halophyte; these are generally not as salt-tolerant as dicotyledonous species, and have little positive response to salinities above 10 mM. Their growth response is not very different from that of wheat over the lower salinities. Typical of a monocotyledonous halophyte is *Diplachne fusca* (also called *Leptochloa fusca*, and Kallar grass), used for forage on saline lands; its growth at salinities up to 250 mM NaCl is little better than wheat (not shown on Figure 2, for clarity), but it can continue growing at salinities in which wheat can not. For example, after two months at 300 mM NaCl the growth of *Diplachne* was 25% of controls, and at 400 mM NaCl was still 10% of controls (Sandhu *et al.*, 1981).

13.2.3 WITHIN-SPECIES DIVERSITY

It is not easy to quantify differences in salt tolerance between species, and it is even harder to quantify differences within species. All the same, diversity in salt tolerance between cultivars or genotypes, assessed as biomass production or survival after a period of time in saline conditions, has been shown within many species in publications too numerous to list here. Perhaps the greatest cultivar difference is found within rice. As an example, the salt tolerance of seven cultivars grown for one month in similarly saline conditions (80-100 mM Cl^- salts with adequate Ca^{2+}) varied from 25% to 76% (Grieve and Fujiyama, 1987; Muhammed *et al.*, 1987; Aslam *et al.*, 1993). Aslam *et al.* (1993) named this method of growing plants for a month in saline soil "a rapid screening technique". They confirmed the validity of this technique in predicting genotype differences in yield in the field.

When screening for salt tolerance amongst a large number of genotypes within a species, it may not be feasible to assess salt tolerance itself; that is, it may not be feasible to grow a large number of plants under both saline and non-saline conditions. In those cases, one of the physiological traits that confer salt tolerance can be used. The trait that has proved most generally useful is a low rate of salt accumulation in leaves.

For a number of species it has been shown that variation in the rate of Na^+ or Cl^- accumulation between genotypes correlates quite well with variation in salt tolerance, as assessed by biomass production or survival in saline soil over a long period (for examples, see review by Greenway and Munns, 1980). This trait, of low Na^+ or Cl^- accumulation in leaves, is conferred by the ability of roots to maintain a low net uptake of salt from the soil, or to restrict loading of the xylem. The ability to compartmentalize salt in cell vacuoles, and so tolerate high tissue levels of salt, is presumably an equally important trait, but is not as easy to quantify. Other traits could be important, such as high growth rates (which will reduce the rate of salt accumulation per unit biomass), high shoot: root ratios, and low transpiration or water use ratios. When Yeo *et al.* (1990) tested a wide range of rice germplasm, the correlation of any one trait with "salinity resistance" (a survival-based index) was found to be low. These authors observed that it was common for rice varieties which were strong in one trait to be poor in others, and suggested that individual traits rather than salinity resistance should be used to select parents for a breeding program.

TABLE 1. The effect of salinity on plant growth at different time scales over which the effects can be measured, the type of long-distance signal or state that could be regulating growth, the cellular events involved, and whether it operates in the water stress or salt toxicity phase. The species indicated as 'sensitive' have poor exclusion by roots or compartmentation within leaves, although this would occur in all species if the salinity was high enough. See text for description.

	Observed effect on growth	Cellular events	Regulating signal or state	Cause
Seconds to minutes	Instant reduction in leaf and root elongation rate then rapid partial recovery	Shrinkage of cell volume then restoration due to regaining turgor	Hydraulic	Water stress
Hours	Steady reduced rate of leaf and root elongation	Changed rheology of cell wall	Hormone (if turgor regained)	Water stress; Ca^{2+} deficiency
Days	Reduced rate of leaf emergence; increase in root : shoot ratio	Cell production rate and primordia development inhibited	Hormone and/or sugar	Water stress; Ca^{2+} deficiency
Weeks	Reduced branch or tiller formation	Apical development program altered	Hormone and/or sugar	Water stress
Weeks – sensitive species	Old leaves die	Na^+ and/or Cl^- accumulates excessively in cells	Ion toxicity or dehydration mature leaves	Ion toxicity
Months	Altered flowering time, reduced seed production	Reproductive development program altered	Hormone and/or sugar	Water stress
Months – sensitive species	Plant dies before maturity	Inadequate capacity for assimilate production to support further growth	Carbon deficit	Ion toxicity

13.3 Growth processes at different time scales

The mechanisms by which salinity affects growth of a plant depend on the time scale over which the plant is exposed to salt. Table 1 summarizes the sequence of events in a plant when exposed to salinity. In the first few seconds or minutes, cells lose water and shrink. Over hours, cells regain their original volume but cell elongation rates are reduced, leading to lower rates of leaf and root growth. Over days, cell division rates are also affected, and contribute to lower rates of leaf and root growth. Over weeks, changes in vegetative development can be seen and over months changes in reproductive development.

The different mechanisms that may come into play are considered below. These mechanisms change with time, from simple hydraulic effects that operate as soon as the roots encounter a saline soil solution, to complex controls at the whole plant level involving long-distance signaling and the supply of assimilates.

Whether hormones regulate the growth rate, rather than water relations or carbon supply, depends on the length of time in saline conditions. In general terms it appears that water relations dominate responses to sudden changes in environmental conditions, hormones determine growth rates at the time scale of a day, and carbohydrates influence growth at the time scale of a week, and affect the ability to produce new organs such as lateral shoots, or reproductive primordia. Salt toxicity affects growth in the longer term; salt toxicity occurs in plants growing in salinities too high for them to adequately control the uptake and compartmentalization of the salt. Salts accumulate in the older transpiring leaves over time, and if accumulating to toxic concentrations, will inhibit growth of the younger leaves by reducing the supply of carbohydrates to the growing cells.

13.3.1 TIMESCALE OF SECONDS TO MINUTES

In leaves and roots there are rapid, essentially instantaneous, changes in expansion rates with a sudden change in salinity. Rapid and transient reductions in leaf expansion rates after a sudden increase in salinity have been recorded in maize (Cramer and Bowman, 1991 a; Chazen *et al.*, 1995), wheat (Arif and Tomos, 1993), rice (Yeo *et al.*, 1991) and barley (Figure 3). In roots also, there are rapid and transient reductions in expansion rates, as indicated by the effect of sudden increases in KCl (Frensch and Hsiao, 1995), and also with sudden increases in sorbitol (Hsiao and Jing, 1987) or mannitol (Frensch and Hsiao, 1995).

Several minutes after the initial decline of leaf and root growth, a gradual recovery is observed that may continue for 30 minutes or more before reaching a new steady rate; this is shown for barley leaves exposed to 60 mM NaCl in Figure 3. With higher concentrations of salinity (80-120 mM NaCl), there may be a lag period as long as 40 min before growth starts to recover, and several hours may elapse before it reaches a new steady rate (e.g. Cramer and Bowman, 1991 a, for leaves; Rodriguez *et al.*, 1997, for roots).

The initial decrease in growth occurs so quickly and so transiently that it would seem to be due to changes in cell water relations alone. That it is purely due to water relations was shown by recent experiments in which leaf water status was maintained as the soil was made saline by a pressurization technique developed by Passioura (1988). Preventing a drop in leaf water status completely prevented the transient changes shown in Figure 3 (Passioura and Munns, 2000; Munns *et al.*, 2000 b).

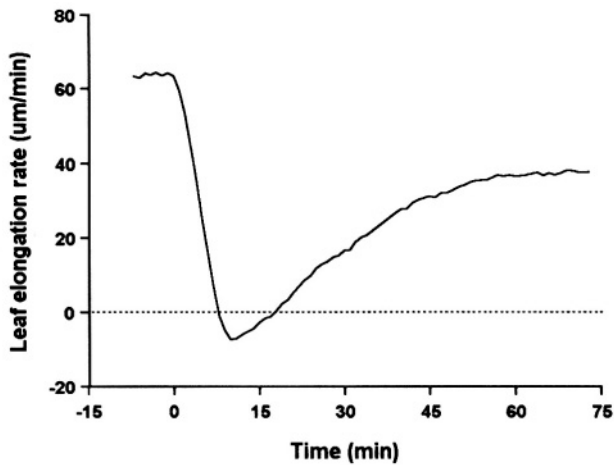


Figure 3. Effect of 60 mM NaCl (added dropwise from concentrated NaCl solution with supplemental Ca^{2+}) on leaf elongation rates of a barley seedling grown in solution culture (R. Munns and R. James, unpublished data).

13.3.2 TIMESCALE OF HOURS

13.3.2.1 *The new steady state*

Leaf growth recovers to a large extent within one or more hours after a sudden change in salinity, settling down to a new steady rate that is considerably less than the original one (Figure 3). The time taken to recover, and the new steady rate, depend on the concentration of the salt solution (e.g. Cramer and Bowman, 1991 a). Other osmotica such as KCl and mannitol have the same effect (Cramer and Bowman, 1991 a; Yeo *et al.*, 1991).

Root growth, in contrast to leaf growth, recovers remarkably well. With moderate levels of osmotic stress (0.1-0.4 MPa of sorbitol, mannitol or KCl), the recovery of growth by maize roots was essentially complete within 1 h (Hsiao and Jing, 1987; Frensch and Hsiao, 1994; Frensch and Hsiao, 1995). With a large osmotic shock (0.6 MPa of mannitol or KCl) there was little or no recovery in elongation rate within 1 h (Frensch and Hsiao, 1995), but it is possible that after 24 h the roots could have recovered. Rodríguez *et al.* (1997) found that growth of maize roots treated with NaCl as high as 150 mM (0.8 MPa) recovered fully after 24 h. These authors compared the

effects of a salt shock versus a gradual increase, and found that salt had no effect on root growth at concentrations up to 100 mM NaCl as long as the concentration was increased gradually. The deleterious effects of a salt shock are presumably associated with the plasmolysis of root cells that would ensue if there was a single-step increase in osmotic pressure of more than 0.4 MPa (as the turgor of root cells is about 0.4 MPa), and the plasma membranes would take some time to repair.

The factors determining the new steady rate of growth are not clear. It is unlikely that salt toxicity is responsible, as other osmotica such as KCl and mannitol have the same effect. Most likely, water relations are responsible. That water relations determines the new rate of growth in the first few hours after an increase in salinity was shown by Cramer and Bowman (1991 b) when they compared leaf growth of maize seedlings whose roots had been excised with those of intact plants, and found they had the same reduction in leaf elongation rate after salinization of the media. These data were confirmed for both barley and maize by Munns *et al.* (2000 b). This finding rules out hormones or other signals produced by roots as controlling leaf growth over this time frame.

Measurements of the turgor of growing cells at this time would help to understand the role of water relations in controlling cell growth, but measurements of turgor in leaf cells is difficult, and almost impossible with leaves of monocotyledonous plants as their growing cells are surrounded by the sheaths of older leaves.

Perhaps the most convincing evidence for leaf water relations controlling leaf growth rates after sudden changes in soil water status is provided by the results of experiments using the pressurization technique mentioned above, in which shoots were maintained at maximum water status while the soil was salinized; not only was the transient reduction in growth rate prevented, but there was no reduction at all for at least several hours (Passioura and Munns, 2000; Munns *et al.*, 2000 b).

13.3.2.2 *The additional influence of calcium*

At this time scale, Ca^{2+} could have an important effect. In a study with two maize cultivars that differed in the requirement for Ca^{2+} , a difference in leaf elongation rate was detected within a few hours of salinization, and was very marked the next day (Cramer, 1992). Furthermore, when the NaCl was removed, the cultivar requiring Ca^{2+} did not recover quickly while the other did.

The influence of Ca^{2+} is particularly important on roots. For example, root elongation of maize was not affected by the addition of 80 mM NaCl provided that supplemental Ca^{2+} (10 mM) was given; when it was not, there was a reduction in root growth that was not readily reversed by subsequent supply of Ca^{2+} (Cramer *et al.*, 1988). Even at 150 mM NaCl, elongation of cotton roots was reduced by only 20% if 10 mM Ca^{2+} was present, but by 40% if it was not (Zhong and Läuchli, 1993).

The mechanisms of the Na^+ - Ca^{2+} interactions are fully discussed in Chapter 10, including the possibility of Ca^{2+} having not only a local effect on cell wall formation, but also a long-distance influence on leaf growth via reduction in hydraulic

conductivity of roots. To prevent deleterious effects of low Ca^{2+} on growth, a minimum of 5-10 mM Ca^{2+} for salinities of 100-150 mM is recommended (Chapter 10).

13.3.3 TIMESCALE OF DAYS

Several experiments suggest that hormonal signals rather than water relations are controlling growth in saline soils over the timescale of 24 h. These experiments were done by growing plants in sand in pots that could be placed in pressure chambers, watering with saline solution, and then pressurizing the chambers with a pressure equal to that of the salt concentration, which was usually 100 mM NaCl. Despite this, no lasting effect on growth was found over periods up to 8 days, with species as diverse as barley and wheat (Termaat *et al.*, 1985), white lupin and Egyptian clover (Munns and Termaat, 1986) as well as a halophyte, the saltbush *Atriplex spongiosa* (Munns, 1993). Lupin is among the most salt-sensitive of plants, and saltbush is a halophyte, so this response may be universal. More recently, these experiments were repeated with plants at balancing pressure, that is, with a pressure that essentially counteracted the leaf water deficit, so that the leaf water potential was close to zero (Munns *et al.*, 2000 a). The growth of barley plants in 100 mM NaCl was still not increased by elevated water status (except when the experiments were run at a high daytime vapour pressure deficit, as described below).

Hormonal signals also appear to be controlling leaf expansion of plants in dry soils, as there was no response of growth to an increase in leaf water status administered in a similar way as described above, that is by allowing the soil to dry, and keeping the shoots fully hydrated by applying sufficient pressure to keep the xylem at atmospheric pressure (Passioura, 1988; Passioura and Gardner, 1990). Further, “split-root” experiments with plants whose root systems were divided between wet and dry soils showed that leaf expansion decreased while leaf water status was unaffected (Saab and Sharp, 1989; Gowing *et al.*, 1990).

Salt-specific effects are again unlikely at this timescale. Not only are there no differences between genotypes observed at this stage, but it is unlikely that salt ever builds up to toxic concentrations in the growing cells themselves. For instance, in the rapidly elongating tissue of leaves of wheat grown in 120 mM NaCl, Na^+ averaged only 15 mM, and Cl^- averaged 50 mM (Hu and Schmidhalter, 1998). The rapid expansion of the growing cells presumably keeps the salt from building up to high concentrations, and in any case one could reason that salt uptake at this stage of cell development would be advantageous for osmotic adjustment.

13.3.3.1 Hormonal control

The hormones regulating growth in dry or saline soils are not known with certainty. Abscissic acid (ABA) is clearly involved in some way. ABA can increase in the growing tissues within hours of a sudden increase in salinity (Chazen *et al.*, 1995), and is at elevated levels in leaves, roots and xylem sap of Nad-affected plants (Zhao *et al.*, 1991; He and Cramer, 1996). As mentioned above, growth reductions occur in leaves whose turgor is unaffected, or whose water status is artificially raised, so there must be

a signal originating from the roots. There is a great deal of evidence for root-sourced ABA regulating growth under drought or salinity stress, as discussed by Davies and Zhang (1991) and Naqvi (1994), although concerns have been raised about the dependence on contributory factors such as pH of the xylem (Bacon *et al.*, 1998), and the possibility of alternative inhibitors (Munns, 1992).

Exactly how ABA might regulate cell expansion is unclear; there is as yet no evidence for it regulating genes that directly control cell expansion. More likely, it may influence the accumulation or operation of other hormones such as gibberellins or ethylene. ABA has been strongly implicated in maintaining root growth under water deficit; roots of ABA-deficient maize plants grew poorly in dry soil, and the addition of ABA could restore this growth (Sharp *et al.*, 1994). It was later found that ABA-deficient roots had higher levels of ethylene than normal roots in dry soil, and elongation could be partially restored by inhibitors of ethylene synthesis or action (Spollen *et al.*, 2000). The finding led to the proposal that a function of ABA synthesis in stressed roots is to prevent an ethylene-induced growth inhibition (Spollen *et al.*, 2000). The role of ethylene is likely to be even more important in saline than in dry soil, as saline soil is very likely to be waterlogged at some stage (Section 4, below).

Gibberellins may be involved in the regulation of cell elongation growth in stressed plants. These hormones control cell elongation under unstressed conditions, but their role in water stress is unknown. The most thorough investigation of the role of gibberellins in stressed plants has been made with water-stressed soybean seedlings, correlating hypocotyl elongation rates with changes in levels of gibberellins (GA) and ABA in the elongating cells following imposition and relief of water stress (Benson *et al.*, 1990). Changes in ABA were more rapid than in GA₁, and linked more closely with changes in growth rate (Benson *et al.*, 1990). These authors concluded that the changes in GA₁ were not of sufficient magnitude nor occurred rapidly enough to be primary regulators of elongation rate responses to rapidly changing water status, we now know that these rapid changes are due entirely to water relations (Passioura and Munns, 2000), so one would not expect hormonal changes to correlate with these sudden changes.

13.3.3.2 *The complication of the diurnal condition of growth*

While the results described above suggest that hormones can override any effect of water relations over the time scale of 24 h, there is evidence that the diurnal pattern of growth may be strongly influenced by water relations. Plants in saline soil show a shift in day:night elongation growth towards the night (e.g. Delane *et al.*, 1982), as do plants in dry soil. We have found that this is reversed by keeping the shoots of plants fully hydrated by applying balancing pressure while the soil becomes dry (Passioura, 1988) or saline (Munns *et al.*, 2000 a). Yet the total growth of plants over 24 h can be unchanged (Passioura, 1988; Munns *et al.*, 2000 a). These results suggest that hormonal signals control growth on a 24 h basis, such that any short-term stimulation of growth arising from unusually high water status during the light period is counterbalanced by slower growth during the night.

In one experiment, however, that was run at a high daytime temperature and a high evaporative demand, total growth of salt-treated plants over 24 h was considerably increased by pressurization (Munns *et al.*, 2000 b). The stimulation of growth during the light period was so great as to be incompletely offset by reduced growth at night, and the growth over 24 h was greater than for the unpressurized plants, although not as great as for the controls without salt. This strongly suggests that leaf water status can limit leaf expansion rates during periods of high transpiration despite the control exercised by hormones on a 24 h basis. This is consistent with field studies with maize in dry soil, which indicate that water relations are dominating growth during the day at high evaporative demand, but hormones appear to be dominating at night (Ben Haj Saleh and Tardieu, 1997). ABA was considered a likely candidate for this role (Ben Haj Saleh and Tardieu, 1997), but the experiments with balancing pressure mentioned above (Munns *et al.*, 2000 a) indicate that the controls are more complex than this.

13.3.3.3 *Developmental changes*

By the time a plant has been in saline conditions for a few days, there is a complete turnover of cells in the growing regions. Rates of cell division may be reduced (Lazof and Bernstein, 1999), so the total number of growing cells may be reduced as well as the rate at which they expand, thus shortening the elongation zone (e.g. Bernstein *et al.*, 1993). The rate of leaf emergence and primordia formation decreases (Grieve *et al.*, 1993; Lazof and Bernstein, 1999). There also may be increases in the root: shoot ratio (reviewed by Munns and Cramer, 1996), suggesting a differential effect on cell division or primordia formation in roots versus shoots.

The hormones most likely to be involved in these changes in rates of cell production or primordia development are cytokinins and auxins. A decreased supply of cytokinins from roots to leaves could inhibit cell division in leaves of water-stressed plants, and influence the root:shoot ratio, as cell division in roots can be promoted by concentrations of cytokinins that inhibit cell division in leaves (reviewed by Munns and Cramer, 1996). Auxins are essential in promoting progress through the cell cycle, but cytokinins are most stringently required at the initiation of mitosis (Zhang *et al.*, 1996). Cytokinins could act directly on kinase or phosphatase modifiers of the key cell cycle enzyme Cdc2, as indicated for leaves of water-stressed wheat (Schuppler *et al.*, 1998). A contributory role of ABA cannot be ruled out. Although the mechanism of action is unknown, studies with mutants deficient in ABA suggest that this hormone is involved in the regulation of root: shoot ratio (Munns and Cramer, 1996).

13.3.4 **TIMESCALE OF WEEKS**

After a week or so, developmental changes appear in the shoot. The number of lateral shoots or tillers is strongly reduced. For example, the number of tillers of wheat growing in 150 mM NaCl was reduced by two thirds (Nicolas *et al.*, 1993). In cereals, and possibly all annuals, salinity can bring forward the time of floral initiation. Even though the rate of leaf primordia production is reduced, the commitment of a given primordium to leaf or floral development is altered, and floral initiation can occur many days earlier (e.g. Maas and Grieve, 1990).

The way that salinity affects these developmental processes is unknown. It is unlikely to be by an effect of Na^+ or Cl^- in the apical tissues themselves, as concentrations there are probably quite low. Salt concentrations in the vegetative shoot apex of lettuce grown in 80 mM NaCl were found to be very low; X-ray microanalysis revealed that Na^+ and Cl^- levels were only 20 mM (Lazof and Läuchli, 1991). Furthermore, similar developmental events are initiated by drought. The developmental changes are more likely regulated by long-distance hormonal or carbohydrate-modulated signals (Koch, 1996), or directly influenced by the supply of carbohydrate as a substrate.

The likelihood of carbohydrate acting as a signal or substrate is supported by the findings that elevated CO_2 increases the growth of plants in saline soil (reviewed by Munns *et al.*, 1999). For example, high CO_2 increased the number of tillers of wheat growing in 150 mM NaCl by 55%, and thereby increased plant growth by 55%, there being no effect on the dry weight of individual tillers or leaves (Nicolas *et al.*, 1993). These results suggest that the flux of carbon to the apex is influencing development; whether it acts as a signal and modulates gene expression (Koch, 1996), or simply provides more carbohydrate substrate to rate-controlling enzymes is difficult to know, but the fact that sugar levels are elevated in these growing tissues (Munns and Termaat, 1986; Nicolas *et al.*, 1993) suggests that the rate of delivery of the incoming carbohydrate is acting as a signal.

Further changes are seen in sensitive species. In this timescale of weeks, the sensitive genotypes will show marked injury in older leaves. It is due to salts accumulating in transpiring leaves to excessive levels, exceeding the ability of the cells to compartmentalize salts in the vacuole. Salts then rapidly build up in the cytoplasm and inhibit enzyme activity, or they build up in the cell walls and dehydrate the cell (Flowers and Yeo, 1986). Salts eventually build up to high concentrations in the transpiring leaves and cause premature senescence. This marks the transition from the first to the second phase of the growth reduction (see Figure 2).

13.3.5 TIMESCALE OF MONTHS

After a month or so, there can be obvious effects of salinity on the development of reproductive organs. Salinity reduced the number of florets per ear in barley and wheat (Munns *et al.*, 1988; Grieve *et al.*, 1994), increased sterility, and altered the time of flowering and maturity in wheat and rice (Maas and Poss, 1989; Khatun *et al.*, 1995; Munns and Rawson, 1999). Similar phenomena occur under drought (see also Chapter 14).

13.3.5.1 Formation of reproductive organs

The mechanisms by which salinity might affect the formation of reproductive organs are not clear. It is probable that signals are hormonal, or are carbohydrate-modulated, affecting the expression of genes switching on developmental programs. Alternatively, the changes could be influenced by the supply of carbohydrate as a substrate; it is difficult to design experiments to separate these possibilities. A salt-specific effect is an unlikely cause of altered reproductive development; Na^+ and Cl^- is present in the

reproductive primordia, but at concentrations too low to affect metabolism. In the reproductive apex of a salt-sensitive cultivar of barley during ear development, Na^+ increased to a maximum of 50 mM at the time of final spikelet initiation, while Cl^- remained low at 10-15 mM (Munns *et al.* 1988). Similar results were found with four other barley and wheat cultivars with known differences in salt tolerance (Munns and Rawson, 1999). It is unlikely that these Na^+ levels or Cl^- levels are toxic to metabolism: only Na^+ levels above 100 mM are known to affect enzymes, and then only if the K^+/Na^+ ratio falls below 1:1.

13.3.5.2 Fertility

The mechanisms by which salinity might affect fertility or seed set are even less clear. In salt-tolerant plants, in which salt has not built up to toxic levels in leaves, and in which there is probably little salt translocated in the phloem to the floral organs, the signals again are probably hormonal or influenced by carbon supply. That is, the situation is the same as for drought. The cause of drought-induced sterility in cereals is the failure of pollen to mature, with meiosis being the most sensitive stage (Saini, 1997). Studies with wheat and rice showed that the stage of pollen meiosis is preceded by a reduction in invertase activity that results in reduced starch synthesis and pollen abortion (Dorion *et al.*, 1996; Sheoran and Saini, 1996). The reduction in invertase activity is probably triggered by a low flux of carbohydrate to the anthers, possibly acting as a signal affecting sugar-modulated gene expression (Dorion *et al.*, 1996; Sheoran and Saini, 1996). ABA might have a role either directly as a signal transported in the phloem from leaves to the reproductive tissues (Saini, 1997), or indirectly in affecting carbohydrate flux as a result of stomatal closure in leaves that supply sucrose to the reproductive organs.

In salt-sensitive plants, in which salt has built up to excessive levels in leaves and the vacuoles can no longer contain the incoming salt, high levels of salt in the cytoplasm or walls may result in translocation of significant amounts of salt in the phloem to the reproductive organs. High levels of Na^+ were found in pollen and stigmas of rice grown at 50 mM NaCl, and stigmatic receptivity was reduced as well as pollen viability (Khatun *et al.*, 1995). These authors concluded that the high degree of sterility was probably due to Na^+ toxicity in the reproductive tissues. This may be peculiar to rice, and explain why the yield of rice is particularly sensitive to salinity: the grain yield was only 10% of controls, whereas the straw weight was 80% of controls (Khatun *et al.*, 1995).

In the sensitive species also, leaves are dying at a fast rate. Salt stress results in a progressive loss of the older leaves with time. The rate at which they die becomes the crucial issue determining the survival of the plant. If new leaves are continually produced at a rate greater than that at which old leaves die, then there is enough photosynthetic surface for the plant to produce flowers and seeds. However, if the rate of leaf death exceeds the rate at which new leaves are produced, then the proportion of leaves that are injured starts to increase. There is then a race against time to initiate flowers and form seeds while there is still an adequate number of green leaves left to supply the necessary photosynthate.

13.4 Waterlogging and salinity

As salinity is caused by saline groundwater rising close to the surface, waterlogging and salinity often occur together. Waterlogging affects plant response to salinity, because oxygen deficiency in the root zone inhibits oxidative phosphorylation; this restricts the energy available for the carriers and pumps involved in controlling the uptake and efflux of Na^+ . The energy demand for salt exclusion and compartmentation is not large in terms of the total energy demand of a rapidly growing plant (Yeo, 1983). However, under hypoxic conditions even this small demand may not be met, and greater net uptake of Na^+ will occur. Na^+ and Cl^- uptake by leaves of wheat in saline soil was doubled by waterlogging (Barrett-Lennard *et al.*, 1999), and in maize K^+ uptake was also affected (Drew and Läuchli, 1985). Thus, waterlogging hastens the onset of the second or salt-specific phase of the growth response.

Additionally, there may be signals affecting growth. These undoubtedly involve ethylene, which accumulates in both roots and leaves of plants in flooded soil (reviewed by Jackson, 1993). Split root experiments showed that a message moves from roots to leaves; this message is probably the ethylene precursor ACC (1-amino-cyclopropane-1-carboxylic acid) (Jackson, 1993). If ethylene is the hormone that inhibits root growth in dry soil, and the function of ABA is to suppress ethylene synthesis, then roots in saline soil that is not well drained will be doubly disadvantaged, as ethylene produced by roots cannot diffuse away as fast as in aerated soil.

13.5 Conclusion

Salinity can affect growth in a number of ways. First, the presence of salt in the soil reduces the ability of the plant to take up water, and this quickly causes reductions in the growth rate. This is the first phase of the growth response, due to the osmotic effect of the salt in the soil solution, and produces a suite of effects identical to those of water stress caused by drought. If the concentration of Ca^{2+} in the soil solution is not elevated above that needed for non-saline conditions, a Na^+ -induced Ca^{2+} deficiency can occur, which will quickly cause an additional reduction in the growth rate. Later, there may be another additional effect on growth; if excessive amounts of salt enter the plant they will eventually rise to toxic levels in the older transpiring leaves. The reduced photosynthetic capacity of the plant will reduce the amount of assimilate transported to the growing tissues, which may further limit growth. This is the second phase of the growth response, and is the phase that clearly separates species and genotypes that differ in the ability to tolerate saline soil.

Despite diversity between species in the ability to grow and reproduce at high salinity, all species suffer marked growth reductions. To answer the question of what extent do hormones regulate growth over the obvious alternatives of water relations and carbon supply, is quite difficult. It appears that:

- (i) in the timescale of minutes, water relations dominate, and may do so during daylight hours particularly under high evaporative demand,
- (ii) in the timescale of days, hormonal (or carbohydrate-modulated) signals control growth,
- (iii) in the timescale of weeks to months, hormonal signals control growth of the salt-tolerant plants in which salts have not accumulated to toxic levels, but in salt-sensitive plants whose older leaves may be dying fast due to excessive accumulation of salts, a reduced supply of carbon to the growing tissues may limit the production of new leaves or even seeds.

This implies that any improvement in drought resistance would make a plant more adapted to saline soil. However, the processes that adapt a plant specifically to saline soil involve the regulation of the uptake and compartmentation of sodium, to delay as long as possible the time when it accumulates to toxic levels in leaves that are actively photosynthesizing. Breeding or genetic engineering of plants better adapted to saline soil should focus on these processes.

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13.6 References

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CHAPTER 14

THE ADAPTIVE POTENTIAL OF PLANT DEVELOPMENT: EVIDENCE FROM THE RESPONSE TO SALINITY

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Abstract

Tolerance to salinity varies throughout plant development. Stable phenophases with a relatively-defined level of tolerance are interspaced with transition periods during which, in some species, an increase in tolerance may be achieved. This adaptive response involves changes at many levels of biological organization, from gene expression to inter-organ relationships. They include overexpression of protective proteins, control of Na^+ uptake, changes in photosynthetic metabolism, restructuring of the hormonal balance and modification of the cellular sensitivity to growth factors. Individuality is endemic to this adaptive response, and results from an extreme sensitivity to environmental and developmental factors during its maturation. For this reason, the adaptive response is interpreted as an expression of the nonlinear dynamics characterizing the transitory period between two stable developmental phases. Accordingly, plant response to salinity is reconsidered as the mutual expression of a pre-existing (genetic) component and the self-organized dynamics of its rearrangement.

14.1 Towards a non-linear approach of plant response to salinity

Exposure to salinity induces modifications at all levels of biological organization, from gene expression to whole plant physiology and development. Hundreds of reports are focused on a given change, comparing the response of salt-tolerant and salt-sensitive species or genotypes. However, it remains difficult to integrate all these modifications into a general framework of plant response to stress. Concerning salt tolerance, a cellular basis is observed in some species but not in others, and growth inhibition is not always proportional to the accumulation of Na^+ and Cl^- ions in the shoot (see below). It seems, therefore, that there are many ways to cope with presence of sublethal concentrations of NaCl in the root medium, disproving any unifying scheme for tolerance. Nevertheless, plant tolerance is assumed to be a stable function of the genetic

information and the regulation of its expression. This representation may fit studies about vertebrates, however, it cannot be easily applied to plants because, in the latter, growth is integrated into extended development. Transformation of the heterotroph embryo into a photosynthetic seedling, or transition to reproductive development in the shoot meristems are the best-known evidence for the phasic nature of development. However, other phenophases are also described. In many plants, juvenile and adult vegetative phenophases are identified by gradual or discrete changes in leaf shape (Ashby, 1948; Schaffalitzki de Muckadell, 1954; Brink, 1962; Telfer and Poethig, 1994). In *Sorghum bicolor*, adventitious root formation is not a continuous event, but periodic short phases of emergence of new adventitious roots may be identified (Blum *et al.*, 1977). In wheat, optimal temperature for growth varies according to the phase of development: there is a long developmental phase from seedling growth to anthesis, followed by several short but distinct phenophases characterized by different sensitivity to temperature (Slafer and Rawson, 1994, 1995).

Modifications in the sensitivity to NaCl during development are a well-known phenomenon in crop plant physiology. Strogonov (1964, p. 13) has reported for a long time that salt tolerance varies during ontogenesis. This is confirmed by recent observations. An increase in tolerance to NaCl is observed during vegetative growth in *Beta vulgaris*, cotton, rice (reviewed by El-Saidi, 1997). In soya, callus cultures from germinating seedlings or late vegetative plants show a similar tolerance to NaCl, but tolerance to NaCl is increased throughout development (Bourgeais-Chaillou *et al.*, 1992). This indicates that a whole-plant level of tolerance is progressively matured during vegetative development. In *Melilotus segetalis*, tolerance to NaCl increases as the plant reaches the reproductive stage (Romero and Maranon, 1994). This phenomenon has been related to an increase in root selectivity for mineral nutrition normally occurring towards reproductive development (Romero *et al.*, 1994).

Beyond this general increase in tolerance during development, agronomists know that phases of tolerance may be interspaced by critical periods of high sensitivity which limit the final yield. In rice, for example, viability of the pollen conditions the final yield for plants exposed to moderate salinity (Kathun and Flowers, 1995). A critical stage of high sensitivity is frequently observed during flower bud formation (El-Saidi, 1997; Dhingra and Varghese, 1997), and this even for plants showing a high level of tolerance during the late vegetative development. Furthermore, salt-tolerance is not a static property specific to each phenophase. Strogonov (1964, pp. 235-236) reported many cases of increase in salt-tolerance following pre-exposure to a moderate level of salinity during the early vegetative development. This author also reported an increase in salinity tolerance for plants exposed to NaCl during embryo maturation or seed imbibition.

Recent work confirmed some of the observations reported by Strogonov (reviewed by Amzallag and Lerner, 1995). For example, exposure of young seedlings of *Sorghum bicolor* to a sublethal NaCl concentration (150 mM NaCl) for three weeks induces an ability to grow at 300 mM NaCl, an otherwise lethal concentration for non-pretreated plants (Amzallag *et al.*, 1990 a). This induced tolerance is accompanied by physiological changes, such as a reduced accumulation of Na^+ , but also by a reduced sensitivity of growth to the Na^+ accumulated in the shoot (Amzallag, 1997 a). Such an increase in salt-tolerance, also termed *physiological adaptation to salinity* (abbreviated as salt-adaptation), is achieved only for pretreatment starting between the fifth and tenth

day following germination (Amzallag *et al.*, 1993). This suggests that, in *Sorghum bicolor*, a developmental window of competence opens during a specific phase of the early vegetative development. Salt adaptation is inducible at various degrees among different *Sorghum* genotypes (Amzallag *et al.*, 1993). However, the capacity to increase tolerance to salinity is not related to the "constitutive" tolerance to salinity of the genotype (Amzallag and Lerner, 1994). The increase in tolerance appears, therefore, as an environmentally-induced response with adaptive value.

A similar critical period for induction of adaptive changes was observed in other species following exposure to a sublethal salinity treatment. In *Phaseolus vulgaris*, an enhanced tolerance to salinity was induced for plants exposed from imbibition, as compared with plants initially exposed few days later (Montero *et al.*, 1997; Table 1). In tomato, beginning the salinity treatment (140 mM NaCl) during germination significantly increased yield, as compared with that of plants initially exposed 20 days later to the same NaCl treatment (Bolarin *et al.*, 1993). As described in *Sorghum bicolor* (Amzallag and Lerner, 1994), the amplitude in expression of this adaptive response varies among tomato cultivars (Bolarin *et al.*, 1993). In seeds of tomato moistened in a solution of 6 M NaCl for three days, a significant increase in leaf accumulation of organic solutes was observed for plants exposed to 70 or 140 mM NaCl, and those even 60 days after sowing (Cayuela *et al.*, 1996). This effect is especially interesting because NaCl was removed after imbibition so the salt treatment started only 11 days after sowing (Cayuela *et al.*, 1996). This reveals that exposure to NaCl during imbibition induced long-term adaptive changes.

The combined influence of imbibition and early-vegetative NaCl treatments has been studied in tomato plants by Cano *et al.* (1991). A significant effect of imbibition treatment was observed on fruit yield, shoot Na^+ content and $\text{K}^+:\text{Na}^+$ ratio. Moreover, these authors observed distinct effects of these two salt-treatments. Seedlings initially exposed to 70 mM NaCl at emergence were more tolerant than plants initially exposed 20 days later to the same NaCl concentration. However, the imbibition treatment increased tolerance both for the early and late-treated plants (Table 2). This result suggests that two independent windows for salt-adaptation are transitorily opened during the juvenile development: the first is open during the early germination stages (or even during the late embryo phase of development on the mother plant), and the second is open during the early vegetative development. A similar identification of two distinct windows of competence for salt-adaptation was reported during early vegetative development in *Sorghum bicolor* (Amzallag *et al.*, 1997).

TABLE 1. Influence of developmental stage at initial exposure on response of *Phaseolus vulgaris* to salinity (75 mM NaCl). Data recalculated from Montero *et al.* (1997)

	leaf area (cm^2)	daily CO_2 assimilation	Na^+ content ($\mu\text{mol/gDW}$)	Cl^- content ($\mu\text{mol/gDW}$)
control	293	2.03	4.4	3.7
NaCl from germination	178	0.84	95.6	101.4
NaCl after emergence	154	0.58	265.2	157.8

TABLE 2. Combined effect of imbibition of the seeds in presence of NaCl (0.5 M or 1 M for 36 h) and developmental stage at initial exposure to 70 mM NaCl (germination or 20 days after sowing) on shoot Na⁺ and K⁺ content on day 45, and on final yield in tomato cv GC-72. Data recalculated from Cano *et al.* (1991)

Imbibition treatment	shoot on day 45		final yield	
	Na content ($\mu\text{mol} / \text{gDW}$)	K / Na ratio	total fruits (kg)	mean fruit weight (g)
70 mM NaCl from germination				
no NaCl	530	0.29	1.20	81
0.5 M NaCl	560	0.33	1.20	100
1.0 M NaCl	540	0.40	2.20	78
70 mM NaCl from day 20 after sowing				
no NaCl	810	0.48	0.49	31
0.5 M NaCl	560	0.60	1.55	64
1.0 M NaCl	260	0.84	1.95	63

14.2 The developmental potential of tolerance to salinity

The above-mentioned observations indicate that salt-tolerance differs between phenophases. They also reveal that an adaptive response may mature during specific periods of competence. In the following, increase in tolerance is analyzed in the light of phenomena occurring during transition periods in normal development. In other words, it is asked whether the potential for physiological adjustment to salinity derives from adaptive properties inherent to transition periods in normal development.

14.2.1 CONTROL OF SODIUM ACCUMULATION IN THE SHOOT

The ionic transport activity is not homogeneously distributed within the roots. Such a differentiation is easily revealed when it is accompanied by cell wall extensions increasing the plasmalemma surface, as well as accumulation of mitochondria near these villousities. These parenchyma cells specializing in active transport, defined as transfer cells, are observed in many species around phloem or xylem vascular systems (Pate and Gunning, 1972). Differentiation of transfer cells is stimulated by exposure to NaCl. In the halophyte *Atriplex hastata*, transfer cells are observed in the absorbing region of the roots only for plants exposed to salinity (Kramer *et al.*, 1978). Also in *Prosopis farcta*, differentiation of transfer cells is observed in the hypodermis of roots exposed to at least 10 mM NaCl (Winter, 1988). These observations indicate that differentiation of transfer cells, identified as an adaptive response to salinity (Kramer, 1983), is not constitutive to development of these halophytes.

Transfer cells are easily recognized by internal extensions of the cell wall. However, functional transfer cells may exist in the absence of such modifications of the cell wall and plasmalemma surface. For example, transfer cells are not described in monocots (Pate and Gunning, 1972), but a functional differentiation of cells with improved ion-exchange activity is observed in roots of some monocots grown in presence of NaCl. In maize roots, the xylem parenchyma of plants grown for two weeks in 50 mM Na₂SO₄

shows a significant increase in organelles such as rough endoplasmic reticulum and mitochondria (Yeo *et al.*, 1977). Their selective reabsorption of Na^+ ions from the xylem is suggested by X-ray microanalysis data (Yeo *et al.*, 1977). Also in *Sorghum bicolor*, exposure to 40 mM NaCl enhanced the ATPase activities in the membranes of root cells by about a five-fold range of magnitude, suggesting an improved control of ion cellular concentration and uptake (Koyro *et al.*, 1993). In this case, electron microscopy revealed an increase in the number of mitochondria per cell, and a transfer-cell like structure on the external cell layer of the root (Koyro, 1997). This fact, which enables to explain the high level of control of NaCl accumulated in *Sorghum* (Boursier and Lauchli, 1989; Amzallag and Lerner, 1994), is especially interesting because *Sorghum* is not considered as a halophyte. Thus, this phenomenon may be interpreted as an environmentally-induced change with adaptive values of the developmental pattern of differentiation. In absence of NaCl, activity of transfer cells may be observed in many Leguminosae species through the shoot-to-root retranslocation of radioactive Na^+ ions artificially supplied to a leaf (Levi, 1970; Lessani and Marschner, 1978; Jacoby, 1979; Winter, 1982 a). Providing a constitutive mechanism of control of shoot accumulation of Na^+ and Cl^- ions (Lauchli, 1984), this activity is enhanced by exposure to NaCl. Winter (1982 a) noticed that, in the relatively salt-tolerant species *Trifolium alexandrinum* (Winter and Lauchli, 1982), retranslocation was simultaneously enhanced for all the leaves after 14 days of exposure to 50 mM NaCl, suggesting that this modification occurred for the plant as a whole. A similar phenomenon is observed in *Phaseolus vulgaris*, in which a sudden drop in leaf sodium content occurs simultaneously for the primary and first trifoliate leaf following two weeks of exposure to 48 mM NaCl (Wignarajah *et al.*, 1975).

TABLE 3. Mean and coefficient of variation (CV) of Na^+ content ($\mu\text{mol/gDW}$) in petiole of the five first leaves of *Trifolium alexandrinum* exposed to 50 mM NaCl after full development of the third leaf. Similar fluctuations (with lower range of magnitude) are also observed in leaves. CV ($100 \times \text{SD}/\text{mean}$) are represented in italics. Data recalculated from Winter (1982 a).

leaf	time (days after treatment)				
	5	7	10	14	18
1 (oldest)	729 <i>23.3</i>	900 <i>27.8</i>	625 <i>7.2</i>	1500 <i>40.8</i>	1583 <i>21.0</i>
2	833 <i>9.6</i>	1604 <i>62.3</i>	708 <i>14.6</i>	1845 <i>51.9</i>	1770 <i>25.8</i>
3	1083 <i>13.3</i>	1437 <i>19.2</i>	1062 <i>7.8</i>	1808 <i>36.8</i>	2083 <i>14.7</i>
4	1090 <i>18.3</i>	1666 <i>19.7</i>	1095 <i>7.5</i>	1893 <i>28.5</i>	2460 <i>37.4</i>
5 (youngest)	1100 <i>9.9</i>	1280 <i>16.8</i>	940 <i>19.9</i>	1770 <i>40.6</i>	2580 <i>31.3</i>

Irrespective of the age of the leaf, such an integrated response is even observed for leaves that have unfolded before exposure to NaCl. This interference between developmental and adaptive mechanisms is detailed by Winter (1982 a, b; Winter and Preston, 1982) for *Trifolium alexandrinum* exposed to NaCl. A sudden drop in petiole and leaf Na^+ contents is observed simultaneously for all the leaves, and this in spite of a large variability in Na^+ concentration before and after this day (Table 3). Thus, it is likely that the drop in leaf Na^+ content reflects a physiological change which is regulated by development. A few days later, this drop is followed by a rapid senescence of these transfer cells, which is probably linked to the enhanced activity of sequestration and retranslocation of Na^+ ions (Winter, 1982 b; Winter and Preston, 1982). The authors also observed a progressive differentiation of xylem transfer cells specifically replacing the phloem transfer cells in salt-treated plants. This may be interpreted as an adaptive, non-constitutive, response by the plant. In conclusion, two adaptive responses are expressed in *Trifolium alexandrinum* when exposed to salinity: (i) a short-term adaptive response, generated by a NaCl-induced stimulation of a constitutive retranslocating activity (phloem transfer cells), and (ii) a long-term response, resulting from differentiation of a new function (xylem transfer cells), that replaces the degenerating phloem transfer cells.

The case of *Trifolium* illustrates how complex the link may be between development and adaptation. Furthermore, differentiation of transfer cells during normal development may also be interpreted as an adaptive response, at least in some cases. For example, xylem and phloem transfer cells are observed at the intersection between cotyledon and hypocotyl during early phases of germination in *Senecio* and lettuce (Pate *et al.*, 1970; Gunning *et al.*, 1970). Such a constitutive differentiation of transfer cells ensures nutrition of the germinating embryo in spite of its weak transpiration stream. However, Pate *et al.* (1970) observed that the removing or darkening of a cotyledon prevents both translocation of photoassimilates and differentiation of transfer cells. They concluded that the nutrient-transfer activity from cotyledons is the stimulus for differentiation of the transfer cells. This case reveals how adaptive differentiation and differentiation of adaptive functions are inextricably linked.

14.2.2 CHANGE IN PHOTOSYNTHETIC METABOLISM

Exposure to 100 mM NaCl for five days enhances expression of the PEP carboxylase in leaves of C4-photosynthesis species such as maize, *Chloris gayana* (Shomer-Ilan and Waisel, 1973), and *Sorghum bicolor* (Amzallag *et al.*, 1990 b). Following exposure to salinity, an increase in PEP carboxylase is also observed in CAM species such as *Cakile maritima* (Beer *et al.*, 1975) and *Mesembryanthemum crystallinum* (Ostrem *et al.*, 1987). In the latter case, the transition from C3 to CAM photosynthetic metabolism is observed following 2-3 weeks of exposure to NaCl concentrations above 100 mM NaCl, and accumulation of malate in leaves is found proportional to the external concentration of NaCl (Winter, 1973). Plants also stabilized their leaf Na^+ concentration during this period (Winter, 1973), suggesting that a dynamic equilibrium is progressively achieved between Na^+ accumulation and shoot growth. As for C4 metabolism, the change towards CAM has been interpreted as one of the mechanisms of long-term adaptation of increasing tolerance to salinity (Lüttge, 1993). The induced change in photosynthetic metabolism differs according to leaf age (Winter and Lüttge, 1979). Moreover, the

adaptive change in photosynthetic metabolism is strongly related to whole plant development, as revealed by the specific phase of competence for induction of CAM response in *M. crystallinum* (Cushman *et al.*, 1990; Piepenbrock and Schmitt, 1991). Furthermore, a progressive shift towards CAM photosynthetic metabolism is observed in *M. crystallinum* grown in absence of NaCl (Cushman *et al.*, 1990). Accordingly, Herppich *et al.* (1992) concluded that the increase in PEP carboxylase is developmentally regulated in this species, but that amplitude of this change is modulated by external environment, and especially by NaCl. Interestingly, Na^+ ions (supplied as a micronutrient) are shown to be required for expression of PEPcase in non-stressed plants (Brownell and Crossland, 1972, 1974). It seems, therefore, that exposure to NaCl enhances expression of a metabolic pathway already stimulated by Na^+ ions during normal development (see also Chapter 16).

14.2.3 TRANSPHENOPHASIC EXPRESSION

Proteins specific to a phenophase may be occasionally expressed during another phenophase. This phenomenon is defined here as *transphenophasic* expression. Even during normal development, plants are exposed to strongly modified environments during specific phenophases. For example, in many pluriannual species, flower morphogenesis occurs when buds are exposed to very low temperatures. Similarly, the late embryo phenophase displays tolerance to very low osmotic potentials during the process of seed dehydration. Thus, it is likely that protective mechanisms are expressed during these phenophases. Osmotin (Kononowicz *et al.*, 1992, 1993), germin and dehydrin (Hurkman *et al.*, 1991; Hurkman and Tanaka, 1996) as well as enzymes involved in protein repair (Mudgett and Clarke, 1994) are protective proteins normally expressed during pollen maturation and seed dehydration.

A transphenophasic expression of protective proteins specific to the late embryo phenophase should improve tolerance to extreme conditions (decrease in osmotic potential, increase in ion concentration) similar to those encountered during seed dehydration. Many authors reported, in vegetative tissues of plants exposed to a salt stress, an over-expression of proteins especially abundant during the late-embryo phenophase (Hurkman *et al.*, 1991; Kononowicz *et al.*, 1992; Mudgett and Clarke, 1994; Hurkman and Tanaka, 1996; Chandler and Robertson, 1994; Campalans *et al.*, 1999). Moreover, transgenic studies provide experimental evidences towards involvement of these proteins in salt-tolerance. In yeast, tolerance to high salinity was enhanced after introduction of a tomato gene coding for a protein specific to the late-embryo phenophase (Imai *et al.*, 1996). In rice, Xu *et al.* (1996) showed an increase in salt-tolerance following introduction of a barley gene specific to the late embryo phenophase. Overexpression of an osmotin-like gene from *Arabidopsis* increased tolerance to salinity in transformed potato plantlets axenically cultured (Evers *et al.*, 1999).

A stimulating effect of abscisic acid (ABA) on growth was observed during embryo development (Thompson *et al.*, 1984), seed maturation (Radley, 1976; Barthe and LePage-Degivry, 1993), and tolerance of the embryo to dehydration (Bochicchio *et al.*, 1994; Phillips *et al.*, 1997). Moreover, it is shown that expression of protective proteins in the late embryo is modulated both by ABA and by the change in osmotic

pressure and ion concentration (Galau *et al.*, 1987; Pacheco-Moises *et al.*, 1997; Giordani *et al.*, 1999).

In consequence, ABA should be considered as a growth regulator involved in control of specific phases of development highly tolerant to extreme environmental conditions. Absciscic acid is also involved in plant response to salinity: (i) an increase in ABA content is rapidly observed following exposure to salinity (reviewed by Hartung and Jeschke, 1999), (ii) tolerance has also been correlated to the level of ABA accumulated following exposure to a moderate salt-stress (Bohra *et al.*, 1995; Moons *et al.*, 1995), (iii) expression of protective proteins has been linked to accumulation of ABA in the tissues of plants exposed to osmotic stress (Berna and Bernier, 1999; Giordani *et al.*, 1999). The involvement of ABA both in embryo development and in response to salinity suggests that this hormone is the regulator of transphenophasic expression of protective proteins from the late-embryo phenophase. Such an interpretation enables to understand the large range protective effects promoted by ABA, a phenomenon termed *cross-adaptation* (Boussiba *et al.*, 1975; Johnson, 1984; Kuznetsov *et al.*, 1993).

We may note two conclusions from these considerations: (i) the residual metabolism of ABA inherent to normal development may be stimulated by environmental conditions similarly to its modulation during late-embryo maturation or transition towards bud dormancy, (ii) this abnormal enhancement of ABA metabolism induces a transphenophasic expression of proteins specific to the late embryo phenophase (or bud dormancy). In fact, the situation is even more complex, because sensitivity to ABA seems to be modulated by the environmental conditions (Galau *et al.*, 1987; Benech Arnold *et al.*, 1995), and because the residual metabolism of ABA is not constant throughout plant development. A peak of ABA is observed at a certain stage of berry development in grapes. It is this peak which is both induced a week earlier and amplified for plants exposed to NaCl (Downton and Loveys, 1981). In *Lupinus* grown under salt stress, both ABA metabolism and cellular sensitivity to this hormone are conditioned by leaf age (Jeschke *et al.*, 1997). A link between stress-induced production of ABA and normal development is also observed in *M. crystallinum*. In this species, the ABA content is especially increased for plants exposed to 400 mM NaCl on the sixth week following germination (Thomas and Bonhert, 1993), a period during which an external supply of ABA is able to induce transition to CAM metabolism even in absence of salinity (Chu *et al.*, 1990). According to these observations, it appears that changes in ABA metabolism (as well as in cellular sensitivity to ABA) are modulated both environmentally and developmentally. It is the integration of such a dual mode of regulation which conditions the transphenophasic expression of protective proteins.

14.2.4 ADAPTIVE MODIFICATIONS OF THE HORMONAL NETWORK

Cellular sensitivity to growth factors is conditioned both by developmental and environmental factors (Trewavas, 1982, 1987, 1991). Thus, beyond the dose-effect of hormones, the integrated response of a tissue depends on modulation of its sensitivity to this hormone (Bradford and Trewavas, 1994). This dual mode of response to growth factors is accompanied by a dual regulation of growth. Activity of a meristem is influenced both by the growth factors it is producing (autoregulation) and by the growth factors originating from other meristems or differentiated organs (heteroregulation). It

has recently been suggested that local regulation occurs mainly through dose response, while heteroregulation occurs mainly through changes in cellular sensitivity (Amzallag, 1999 a). Through its redundancy, this dual mode of regulation generates a network of relationships between organs, thus ensuring a high degree of stability of the developing organism despite environmental and developmental variations. On the other hand, this stable network may be adaptively modified throughout specific periods of transition during which the inter-organs relationships are temporarily reduced (Amzallag, 1999 b).

The adaptive property of the network enables to relate some unexpected observations. For example, even though involvement of ABA in tolerance to stresses is clearly demonstrated, mutants with low ABA were shown to be very tolerant to NaCl (Leon-Kloosterziel *et al.*, 1996) or to desiccation (Boccicchio *et al.*, 1994; Giordani *et al.*, 1999). Similarly, cytokinin (CK) and ABA are generally considered as antagonists (Vaadia, 1976), so low cytokinin levels are supposed to be required for expression of the stress response (Badenoch *et al.*, 1996; Goicoechea *et al.*, 1995). Nevertheless, CK and ABA are not opposed to each other in enhancement of the transition to CAM in *M. crystallinum* (Thomas *et al.*, 1992; Schmitt and Piepenbrock, 1992). Furthermore, a tobacco mutant with a CK concentration ten-fold higher than in control plants showed a response to stress similar to that expected for normal plants (Thomas *et al.*, 1995). All these observations confirm that sensitivity to hormones may be adaptively modulated. Moreover, the non-deterministic properties characteristic of complex networks suggest that an adaptive response may be elaborated through many different pathways of modification of the hormonal network.

14.2.5 ADAPTIVE MODULATION OF THE GENOME EXPRESSION

The above-mentioned qualitative or quantitative changes result from adaptive modifications in genetic expression. This phenomenon is also related to events occurring during normal development. Genomic changes, mainly involving amplification/deletion of repetitive DNA sequences, are frequently observed during normal development in plants. These changes are consecutive to cellular differentiation, and they are influenced by the hormonal balance (reviewed by Bassi, 1990). Changes in repetitive DNA content have been also observed during specific transitory periods of normal development, such as transition to the late embryo phenophase (Cavallini *et al.*, 1989), germination (Nitsan and Lang, 1966; Chen and Osborne, 1970; Miksche and Hotta, 1973; Brockaert *et al.*, 1979; Frediani *et al.*, 1994), transition from juvenile to adult vegetative phenophase (Altamura *et al.*, 1987), and transition from vegetative to reproductive development (Wardell and Skoog, 1973; Jacqmard *et al.*, 1981). This suggests that critical periods of transition between two phenophases are characterized by genetic instability. Amplification / deletion of repetitive DNA sequences is not random (Frediani *et al.*, 1994; Ceccarelli *et al.*, 1997). Nevertheless, large variations in DNA content were reported, even between homologous differentiated cells from the same individual (Rogers and Bendich, 1987). This indicates that DNA transactions inherent to differentiation are not completely pre-determined.

Highly-repetitive DNA sequences are not transcribed, so they are frequently considered as "parasitic DNA". It is probably the reason why these developmentally-regulated DNA transactions have been so ignored. However, the repetitive DNA conditions the

spatial conformation of the genome (Amzallag, 1999 c), and especially its level of superhelicity (Kanaar and Cozzarelli, 1992). The tertiary structure of the genome is known to influence gene transcription (Stoilov *et al.*, 1989; Lee and Garrard, 1991; Travers, 1992). At the whole plant level, the importance of DNA transactions is revealed by (i) the relationship observed between repetitive DNA content and phenotypic characters such as rate of germination and growth, stem and culm height, and (ii) the positive correlation observed between genome size and environmental conditions in natural population (reviewed by Amzallag, 1999 c).

Environmental conditions have been known to induce modifications in the repetitive DNA content (reviewed by Cullis, 1990, 1999; Bassi, 1999), and those during specific transition periods of development (Durrant and Jones, 1971; Cullis and Charlton, 1981; Natali *et al.*, 1993; Ceccarelli *et al.*, 1997). As suggested by individuation in amplification / deletion of repetitive DNA sequences, it is likely that these environmentally-induced changes are not pre-programmed. They are rather the expression of a thermodynamic dissipation of constraints in regulation of gene expression (Nagl 1983; Hearst and Hunt, 1991; Kanaar and Cozzarelli, 1992; Vologodskii *et al.*, 1992). This "thermodynamic resolution" of the topological constraints involves nonequilibrium transition phases (Popp and Nagl, 1986), so this process is both adaptive and extremely sensitive to environmentally-induced modifications in genome expression.

14.3 Developmental integration of the adaptive responses

Tolerance to NaCl is frequently associated with a capacity to reduce Na^+ accumulation in growing and physiologically-active tissues. However, shoot Na^+ interferes with rate of growth through: (i) the capacity of control of shoot Na^+ concentration, (ii) the level of sensitivity of the tissues to Na^+ accumulation, (iii) the use of Na^+ ions for shoot osmoregulation. This may explain why no general conclusion may be reached concerning Na^+ toxicity as the limiting factor for growth. A negative correlation between shoot Na^+ accumulation and shoot DW or tolerance may be observed in some cases (see for example Aswathappa and Bachelard, 1986; Schachtman and Munns, 1992; Perez-Alfocea *et al.*, 1993), but not in others (see for example Munns and Termaat, 1986; Yeo and Flowers, 1986; Hajibagheri *et al.*, 1987; Yeo *et al.*, 1990; Glenn *et al.*, 1992; He and Cramer, 1993; Cramer *et al.*, 1994; Amzallag, 1997 a, 1999 d). In *Sorghum*, for example, each one of the three above-mentioned parameters varies independently-throughout development (Amzallag, 1999 d).

The cellular response to salinity may be considered developmentally integrated when parallel tolerance is observed at the whole plant and cellular level. This is the case for the grass *Distichlis spicata* (Warren and Gould, 1982), for *Beta vulgaris* (Smith and McComb, 1981) and for tomato (Cano *et al.*, 1996). Similarly, an increase in salt-tolerance was noticed in plants regenerated from cultured cells of *Medicago media* adapted to grow under conditions of high salinity (Chaudhary *et al.*, 1997). However, no relationship between callus and whole plant tolerance to NaCl has been observed in *Phaseolus vulgaris* (Smith and McComb, 1981), nor has it been observed in many halophytes (Smith and McComb, 1981; Adams *et al.*, 1992; Amzallag, 1997 b; Bajji *et al.*, 1998). In tobacco, salt-tolerance of the regenerated plant is not improved by salt-

adaptation of the cells, in spite of stability of the cellular salt-adapted status following plant regeneration (Wadat *et al.*, 1991). In *Chloris gayana*, the NaCl concentration inhibiting growth of the whole plant stimulates growth of isolated roots (Waisel, 1985). Following a salt-adaptation treatment in *Sorghum bicolor*, the number of seeds per spike varies between complete sterility (early abortion of spikelets or flowers) and 900 seeds (similar to the number of seeds in control plants). However, no relationship can be observed between the number of seeds per spike and size of the shoot (Amzallag, 1998). It has been suggested that strong physiological changes related to salt-adaptation may disturb expression of the next (reproductive) phenophase (Amzallag and Seligmann, 1998). All these examples indicate that, both in glycophytes and halophytes, the cellular component of tolerance is far from always being integrated at the whole plant level. Tolerance to salinity is conditioned by one of the levels of biological organization (molecular, cellular, developmental, or whole plant physiology), but this "determining level" differs among species, individuals, and even among phenophases within an individual.

These considerations reveal that there is a multiplicity of ways to cope with salt perturbation for a plant. This diversity is generated by (i) the nature of constitutive physiological processes and their contribution to tolerance at different levels of organization, (ii) the complex and non-deterministic properties of the adaptive responses, (iii) the capacity to integrate adaptive modifications during transition periods of development.

14.4 Individuation of the adaptive response

NaCl is generally considered as a trigger for expression of a pre-existing program for tolerance. Thus, the existence of critical periods for induction of the adaptive response is interpreted as a developmentally-controlled change in competence for expression of the protective mechanisms. The reality is, however, more complex. In rice, a very large variability in sodium accumulation is observed among genetically-homogeneous populations exposed to a sublethal level of NaCl (Flowers and Yeo, 1981). Contrasting with the relative homogeneity observed in K^+ accumulation, such a variation (including a plurimodal frequency distribution) was not considered as artifactual nor interpreted as the expression of any genetic heterogeneity (Flowers and Yeo, 1981). In *Sorghum bicolor*, a specific increase in phenotypic variability can also be observed during the period of maturation of the salt-adaptation response. Emerging during the three-week pretreatment, this phenotypic variability is proportional to the ability of the genotype to increase its tolerance to salinity by NaCl pretreatment (Amzallag *et al.*, 1995).

Exposure to stress is able to induce noise in expression of a genetic program, resulting in the enhancement of phenotypic variability (Parsons, 1992; Premchand *et al.*, 1998). Indeed, phenotypic variability in reproductive development is also enhanced in *Sorghum* plants firstly exposed to NaCl after closure of the competence window, so that the adaptation response is not initiated. In this case, however, the frequency distribution of the characters measured remains monomodal (as observed for control plants), while it is plurimodal in a population of salt-adapted plants (Amzallag, 1998). This result suggests that the enhanced variability observed following salt-adaptation differs from the noise in

genetic expression generated by exposure to NaCl. In salt-adapted *Sorghum* plants, variability in shoot K^+ concentration is related to microenvironmental variations, but the fluctuations remain very small in regard to those observed for shoot Na^+ concentration (Amzallag, 1999 d). Further analyses revealed three successive stages in evolution of this variability: (i) an initial influence of microenvironmental fluctuations, (ii) an amplification of these variations in a non-linear way, (iii) an emergence of discrete physiological modes of regulation of shoot Na^+ accumulation: Na^+ excluder and Na^+ includer (Amzallag, 1999 d). Furthermore, it seems that it is not NaCl, but rather the environmental perturbation *as a whole*, which is integrated in *Sorghum* development during the critical period of sensitivity (Seligmann and Amzallag, 1995).

All these observations aim for a self-organized source of variability during transition periods in development. This source is initially sensitive to microenvironmental fluctuations, but the biological entity evolves towards discrete adaptive states integrating complex physiological or morphogenetic processes. This is the basis for individuation observed during normal development (Trewavas and Malho, 1997) as well as maturation of an adaptive response to salinity.

14.5 Spontaneous adaptability of the biological systems

An organism in development appears as a dynamic system in perpetual disequilibrium (Ito and Gunji, 1994). By evolving in a narrow range of stability at the "edge of chaos", organisms in development are perturbed in their integrative physiology by emergence of newly-developing structures (Chauvet, 1993 a). This perturbation is able to generate critical phases of instability during which the new structure and the already existing organism are adaptively integrated (Chauvet, 1993 b, 1993 c; Ito and Gunji, 1997; Hielt, 1999). As recently reported for modifications in DNA content (see above), this adaptive property is an expression of the spontaneous evolution of dynamic systems towards the minimum free-energy status (Prigogine and Wiame, 1946; Conrad, 1979, 1990). Thus, development may be understood as a stable phenomenon (phenophase) which becomes close-to-chaotic (transition period) as soon as the level of perturbation overcomes a critical level.

Emergence of a self-organized process requires a temporal decrease of the interrelation between the already-existing structures (Chauvet, 1993 b). Such a drop in connectedness between developing organs is observed in *Sorghum bicolor* during the specific period of aperture of a developmental window for adaptation (Amzallag, 1999 b). A positive relationship exists between decrease in inter-organ connectedness and capacity of adaptation of a genotype (Amzallag, 1999 b). These experimental observations coincide with theoretical considerations, in which non-linear dynamics confer new properties to biological systems, especially individuation and adaptability (Conrad, 1983).

A high level of phenotypic individuality is observed during development at all levels of biological organization (reviewed by Trewavas, 1999). Individuality of neighbor cells for metabolism, gene expression and hormone sensitivity reveals that the phenotype is not the deterministic expression of a pre-existing program of development. Concerning the cellular sensitivity to hormones, Trewavas (1999) noticed that, "If seed gibberellin content is also individualistic, ...then the mechanistic view of growth regulator control is

strongly called into question." In spite of this large range of individuality, growth of different organs is integrated in a species-specific pattern of development. This indicates that the endemic individuality observed at a microdevelopmental level is canalized and harmoniously integrated within macrodevelopment. However, because of the unpredictable nature of the individuality, this integrative process, which occurs during normal development, has *necessarily* an adaptive nature. Thus, beside the pre-existing genetic information encoding for proteins, an adaptive process ensures an harmonized integration of individuality, and this at all the biological levels. This phenomenon was described by Lüttge (1993) as follows: "As a basic intrinsic property all [biological] levels share complexity. It may be rather like the self-resemblance of fractals, in so far each time we change the scaling of the approach or the magnification, we encounter complex interactive functional networks with feedback and non-linear behaviour, i.e. we are confronted with similar theoretical structures."

In consequence, it seems that development results from the conjunction of two phenomena. In the first, growth and microdevelopment are regulated around a defined homeostatic equilibrium. A relatively-strong link may be observed between genetic information and its phenotypic expression during these stable phenophases. In the second, a reorganization of the whole entity occurs according to principles related to dynamics of systems out of equilibrium non-directly related to the expression of pre-existing information. By its relative autonomy, this process also includes an ability of the organism to adapt to external perturbations, such as changes in the environment.

14.6 Conclusion

An analysis of the response to salinity reveals that development is not reduced to a cumulative expression of pre-programmed events. Rather, it seems to be a succession of stable phenophases and brief transition periods with adaptive properties. This view introduces a new dimension into the classical scheme. A phenophase is not only the built-in expression of a specific genetic program activated by internal or environmental triggers, but is adaptively matured during transition periods through complex interactions between an internal information (genetic and structures / functions elaborated during the previous phenophase) and peripheral stimuli (the new emerging organs and/or external changes).

The succession of stable phenophases during development complicates the scheme concerning plant tolerance, due to many reasons. Firstly, adaptive changes in a phenophase may be integrated within the development of the next phenophase, but they may be also disturbing, so final yield cannot be easily expected from expression of the adaptive response. Secondly, if the transition periods are determined by the development itself, their capacity to integrate an environmental perturbation varies considerably, even within a species (Amzallag, 1999 b), independently for each transition period in development. Thirdly, transition periods are not restricted to the whole plant level of organization, but may be found at all the subadjacent levels, from cell-to-cell interactions to DNA transactions. Therefore, it is likely that adaptive adjustments occur at all levels of biological organization, but are not always completely integrated at the whole plant level. This implies that the decrease in growth following a

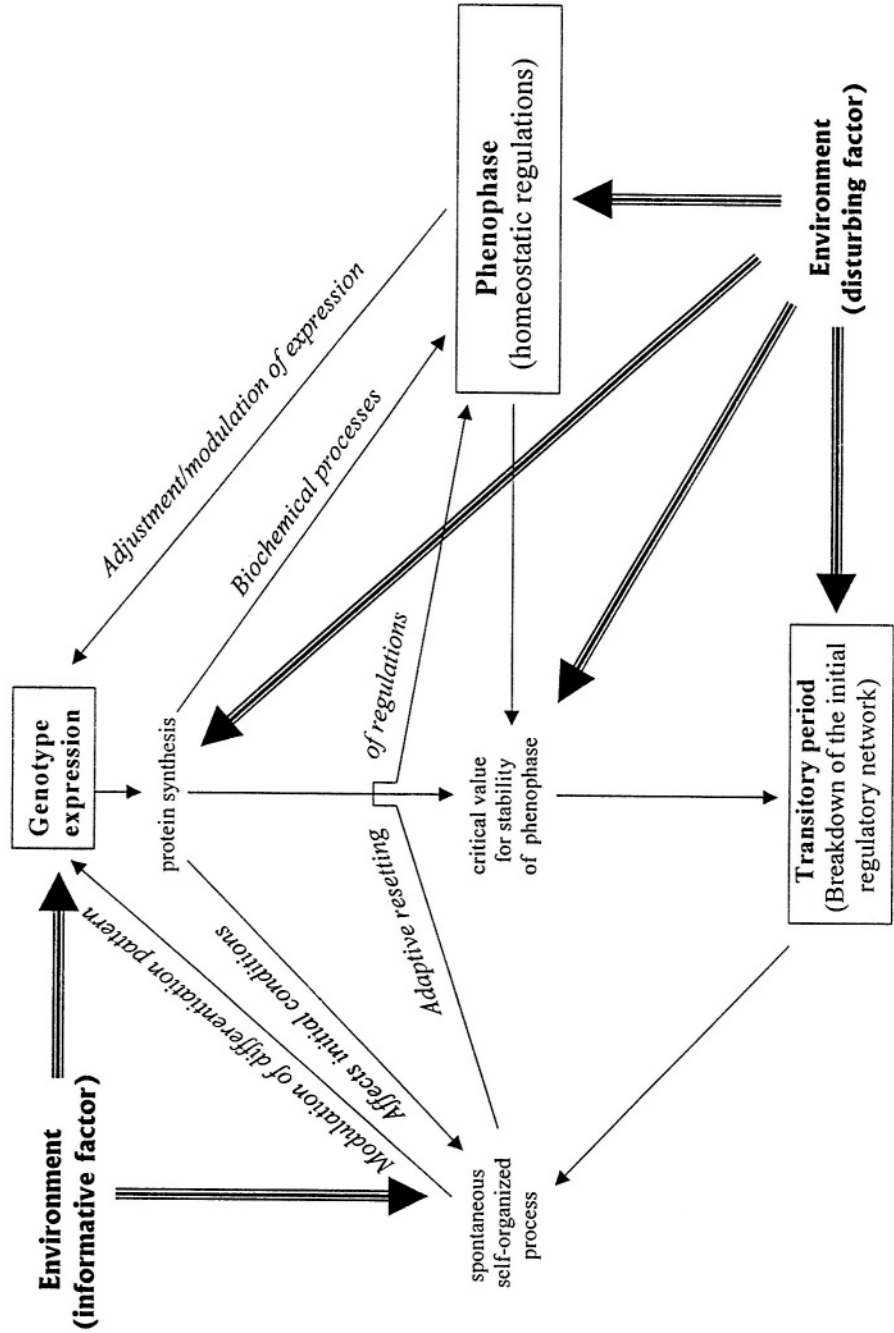


Figure 1: Schematic representation of the role of transitory periods in reorganization of development after perturbation

NaCl treatment may be considered as a parameter of stress only *within* a phenophase. When applied during a transition period, NaCl may be perceived as an environmental stimulus adaptively integrated within the next phenophase through self-organized processes. This "thermodynamic resolution" of the constraint is oriented by viability and not by conservation of the initial equilibrium and rate of growth.

All these considerations beg for a new framework concerning plant response to salinity (and to stress in general), as shown in Figure 1. This framework should be organized both in time around specific mechanisms of defence increasing the tolerance, and around the adaptive properties of non-linear transient dynamics inherent to all biological systems (Amzallag, 2000). Thus, it enables us to understand the developmental origin of adaptive responses as well as their integration in a supramolecular level of organization during transitory periods. Moreover, it even reveals the adaptive component of normal development, which, in general, remains cryptic under optimal conditions.

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14.7 References

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D. PHOTOSYNTHESIS

Photosynthesis is the basis of production of new biomass and growth, and we need to consider salinity effects on photosynthesis not only under the aspect of survival strategies under stress but also under the aspect of productivity (Chapter 15). Special modifications of photosynthesis based on the **C₄-pathway** of primary carbon acquisition, i.e., **CO₂-fixation** via phosphoenolpyruvate carboxylase, as in **C₄-plants** and crassulacean acid-metabolism (CAM) plants, have been considered to be adaptations to stress by drought and high irradiance. These modifications increase water use efficiency and provide mechanisms that reduce risks of photoinhibition and photooxidation. It is an intriguing question to what extent these are also syndromes of traits useful in salinity responses. All three chapters of this section are concerned with this question. While Chapter 15 addresses the basic physiology of photosynthesis under the influence of salinity, Chapter 16 assesses the diversity of C₄- and CAM-plants among halophytes, and Chapter 17 discusses salinity induced expression of CAM. The latter seems to be quite unique in the Aizoaceae-species *Mesembryanthemum crystallinum* L. and perhaps a few very closely related species. Although there are other examples of CAM-induction in **C₃/CAM-intermediate** species regulated by environmental cues, genuine salinity-induced CAM appears to be a very isolated phenomenon in *M. crystallinum* (Chapter 16). Are we witnessing an early new trend in evolution here? In any event, the case study of *M. crystallinum* has become and will be increasingly exciting since it provides the most detailed insights into the molecular aspects of salinity reactions, and stress-reactions in general, available for a higher plant to date (Chapter 17).

CHAPTER 15

INFLUENCE OF SALINITY ON PHOTOSYNTHESIS OF HALOPHYTES

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Abstract

Tolerance of the photosynthetic apparatus of halophytes to saline conditions is brought about by adaptations at a range of scales, from biochemical adaptations to specialized morphologies. Of paramount importance is the capability to minimize water loss through reduced stomatal conductance while maintaining photosynthetic carbon gain. Recent work suggests the capacity to withstand photooxidative stress is also an important component of the suite of traits that confer tolerance of halophytes to saline conditions. Eutrophication of saline habitats, increases in atmospheric CO₂ and rising sea levels are likely to modify photosynthesis of halophytes under saline conditions, and may therefore alter ecological processes.

15.1 Introduction

In contrast to glycophytes, photosynthesis and growth of halophytic plants are maximal in low to moderate salinities (reviewed by Long and Baker, 1986). As agricultural ecosystems become increasingly salinized, which reduces the world's capacity for food production (Chapter 1), attention of researchers has been directed toward plant species that have high tolerances of salinity in order to better understand the physiological basis for salt tolerance (Flowers and Yeo, 1995; Bohnert and Jensen, 1996). It is well established that salinity tolerance is a complex phenomenon, brought about by a range of physiological processes that are under multi-gene control (Bohnert *et al.*, 1995, Chapter 22). In this review, we concentrate on how salinity affects photosynthetic carbon gain in halophytes. Rather than focus on the affects of salinity on component photosynthetic processes, which have been reviewed comprehensively elsewhere (Greenway and Munns, 1980; Munns *et al.*, 1983; Long and Baker, 1986; Cheeseman, 1988), we have taken an integrated approach, recommended by Cheeseman (1988). We first briefly review how salinity affects component photosynthetic processes at the scale

of individual leaves (biochemical and stomatal limitations). Following this, we consider the importance of conservative water use of halophytes during photosynthesis and the special characteristics that facilitate conservative water use in these plants. We then review new evidence which suggests that halophytes may have more well developed mechanisms for dealing with the increased susceptibility to photooxidative damage that results from their low photosynthetic rates imposed by saline conditions than do glycophytes. Next, we move up in scale to consider the relationship between photosynthesis and growth in halophytes. Finally, we review how photosynthesis of halophytes may be affected by the current and emerging influences of eutrophication and global climate change.

15.2 'Biochemical' or stomatal limitations to photosynthesis?

Reduction in photosynthesis in halophytes under saline conditions can be due to a range of factors that have been grouped into two categories, the biochemical capacity of leaves to fix CO_2 , and the diffusion of CO_2 through stomata to sites of fixation. The relative contributions of these two factors to variation in photosynthesis under saline conditions have been hotly debated. Sophisticated analyses of plots of photosynthetic carbon assimilation rates as a function of intercellular concentrations of carbon dioxide (CO_2), called A-Ci curves (Jones, 1973; Farquhar and Sharkey, 1982), allow assessment of the relative limitations of these two factors to photosynthetic rates in intact leaves (reviewed by Ball, 1986, Long and Baker, 1986). Despite the fondness for dichotomies in science, analyses of A-Ci curves have shown that reductions in photosynthetic carbon gain under saline conditions occurs because of both biochemical and stomatal limitations in both halophytes and glycophytes (Ball and Farquhar, 1984; Pearcy and Ustin, 1984; Ziska *et al.*, 1990; Brugnoli and Bjorkman, 1992; Delfine *et al.*, 1998), although there are species (e.g. Pearcy and Ustin, 1984; Brugnoli and Lauteri, 1991), varietal (Kingsbury *et al.*, 1984), and environmental differences (Ball and Farquhar, 1984; Brugnoli and Bjorkman, 1992) in the relative limitations imposed by either factor. For example, Brugnoli and Bjorkman (1992) found in cotton that at moderate salinities (26‰ seawater) limitations to photosynthesis were mainly due to reductions in stomatal conductance, while at higher salinities (55‰ seawater) reductions in photosynthesis were more likely due to changes in the enzymes of the photosynthetic carbon reduction pathway.

15.2.1 IONIC RELATIONS WITHIN THE CHLOROPLASTS

The uptake and distribution of sodium and chloride are regulated to avoid the toxic effects of these ions on metabolic processes. Sodium and chloride are stored in vacuoles (i.e. compartmentalized, Chapter 8) and balanced by compatible osmotic substances in the cytosol (reviewed by Munns *et al.*, 1983; Cheeseman, 1988; see Chapter 9, this volume for a detailed discussion). Earlier work suggested that concentrations of NaCl could be high within the chloroplasts of halophytes (e.g. Critchley, 1982), but later, ion contents within the chloroplasts were found maintained at a constant concentration over a wide range of salinities (e.g. Robinson and Downton, 1985). There is little evidence that chloroplasts are exposed to high concentrations of NaCl in either halophytes or

glycophytes except, perhaps, under severe salinity stress (reviewed by Ball, 1986). Then, disruption of the ionic milieu in chloroplasts would have negative consequences for photosynthesis. For example, two proteins in PSII (a 24 kD and a 17 kD polypeptide) appear to regulate the affinity of the oxygen-evolving complex for chloride and calcium, ions that are important for the maintenance of function of the oxygen-evolving site (Zimmerman and Rutherford, 1985). Accumulation of high NaCl concentrations would cause release of these extrinsic polypeptides from the oxygen-evolving complex of PSII, leading to reduction in oxygen evolution and vulnerability to photoinhibition (Ball and Anderson, 1986). Additionally, high salinities can induce potassium deficiencies within chloroplasts that indirectly disrupt functioning of photosystem II, presumably through effects on protein synthesis (Ball *et al.*, 1987). The sensitivity of photosynthetic membranes of halophytes to NaCl appears to be similar to that of glycophytes. Thus, there is no evidence that chloroplasts are sites of adaptation to high ion concentrations, emphasizing the importance of ion compartmentalization within cells to maintenance of metabolic activity in plants growing in saline habitats.

Nevertheless, beneficial changes in chloroplast structure and function may occur under saline conditions (compare also Chapter 8). Recently, Carter and Cheeseman (1997) observed there was a reduction in thylakoid stacking in grana of chloroplasts of lettuce grown at elevated salinities. In their experiments plants grown at high salinities had relatively unstacked thylakoids compared to control plants grown at low salinity. Li and Ong (1997) made similar observations in gametophytes of the halophytic mangrove fern, *Acrostichum aureum*. Moreover the high salinity grown lettuce in Carter and Cheeseman's (1997) experiments responded to reductions in incident light in a similar way to control plants, by increasing the stacking of their membranes, indicating that the membranes of the lettuce grown at high salinities were indeed functional. The size of granal stacks within the chloroplasts are sensitive to light conditions and are important in the regulation of the capacities for light harvesting and carbon gain. PSI and ATPase are associated with the non-appressed regions of the thylakoid membranes while PSII is found in the appressed regions of the thylakoid membranes (Anderson *et al.*, 1988). Relatively unstacked thylakoids occur in plants grown under high levels of sunlight and are associated with greater levels of exposure of PSI and ATPase to the stroma. Thus, under saline conditions thylakoid membranes appear to be arranged in a manner that could minimize the over-reduction of PSII, and thus the likelihood of photooxidation of PSII and photoinhibition (see below).

15.2.2 "BIOCHEMICAL" LIMITATIONS

The nature of limitations imposed by photosynthetic biochemistry under saline conditions is still under discussion. It has been established that levels of RuBisCO remain constant although rates of photosynthesis decline in plants growing in adverse salinities (Ziska, 1990; Delfine *et al.*, 1998; but see Brugnoli and Bjorkman, 1992, where leaf nitrogen concentrations are reduced in cotton growing at high salinity). Thus, it appears that RuBisCO could be deactivated under saline conditions. This may be due to reduced RuBP regeneration (Ball and Farquhar, 1984; Bonghi and Loreto, 1989; Ziska, 1990) resulting from the accumulation of carbohydrates due to reduced consumption of photosynthetic products for growth (Everard *et al.*, 1994; Gilbert *et al.*, 1997). For example, when the salt tolerant plant, *Coleus blumei*, was exposed to enhanced root

zone salinity, large changes in both the type and amount of carbohydrates exported from leaves were observed (Gilbert *et al.*, 1997). These authors suggest that changes in the synthesis and export of carbohydrates during salinity stress may be important in signaling, and thus may lead to the induction of responses that facilitate survival under saline conditions. Unfortunately comparative studies of carbohydrate metabolism among glycophytes and halophytes, that would allow assessment of whether halophytes function differently in this respect compared to glycophytes are not yet available, with the exception of studies of the C3-CAM switching plant *Mesembryanthemum crystallinum* (Vernon and Bohnert, 1992; Bohnert *et al.*, 1995; see Chapters 16 and 17). In this species, salt stress results in large changes in carbohydrate metabolism that are linked to salinity tolerance (Vernon and Bohnert, 1992; Tarczynski *et al.*, 1993; Shen *et al.*, 1997).

Reductions in cell wall and liquid phase conductance to CO_2 may also result in reduced rates of photosynthesis, although this was not considered to be an important limitation to the “biochemical” component of photosynthesis in earlier analyses. Environmental stresses that induce changes in leaf anatomy, such as thickening of cell walls, can restrict CO_2 diffusion toward chloroplasts (Evans and von Caemmerer, 1996). This causes CO_2 concentrations at carboxylation sites to be substantially lower than in intercellular air spaces, thereby favoring photorespiration. Under saturating irradiance, changes in the CO_2 concentration within chloroplasts would alter the relative rates of CO_2 assimilation and photorespiration for the same level of RuBisCO activity (Evans and von Caemmerer, 1996). Thus salinity-induced changes in cell wall conductance might explain the decline in assimilation rates in the absence of a change in extractable RuBisCO activity under salinity stress.

To date, analyses of gas exchange characteristics indicate a high level of variability in mesophyll conductance to CO_2 . Epron *et al.* (1995) observed that woody species have higher internal resistance to CO_2 than herbaceous species. This resistance could be on the order of 27-32% and the same order of magnitude as imposed by stomata. Decreases in mesophyll conductance have been observed in glycophytes under saline conditions (Bongi and Loreto, 1989; Delfine *et al.*, 1998). Bongi and Loreto (1989) speculated that decreased mesophyll conductance was due to morphological changes in mesophyll cells that limited CO_2 diffusion. In spinach exposed to saline conditions, Delfine *et al.* (1998) found that reduction in mesophyll conductance was also associated with changes in leaf morphology. They suggested that reduced amounts of intercellular space increased the tortuousness of the path of CO_2 to chloroplasts, thereby increasing the diffusional resistance to CO_2 under saline conditions. This would cause a decrease in assimilation rate independent of decreases in photosynthetic capacity.

Morphological changes (Chapter 7) also occur in leaves of halophytes under salinity stress. For example, leaf thickening occurs in the salt marsh grass *Spartina alterniflora* (Hwang and Morris, 1994; Hester *et al.*, 1996; Hester *et al.*, 1998) and in woody mangrove species (Ball, 1996) when exposed to high salinities. However whether such morphological changes are associated with alterations in mesophyll conductance in halophytes has yet to be thoroughly investigated. In a possibly analogous situation, chestnuts varying in drought tolerance were observed to have differences in mesophyll conductance to CO_2 , with higher conductance in drought tolerant compared to drought sensitive varieties (Lauteri *et al.*, 1997). In this case, increased mesophyll conductance

was associated with decreased leaf thickness (a specific leaf area of 81.7 in drought sensitive genotypes to 101.3 g m⁻² in drought tolerant genotypes) and increases in leaf soluble proteins.

15.2.3 STOMATAL LIMITATIONS

Stomatal limitations to photosynthesis under saline conditions do not arise due to reduced leaf turgor, as was originally thought, but is a highly regulated response to salinity (Munns, 1993). The movement of stomatal guard cells and the rate of photosynthesis are tightly coordinated, such that stomatal aperture is constrained within a range where the rate of photosynthesis is at a maximum, while water loss is maintained at a minimum value (Ball and Farquhar, 1984; reviewed by Jarvis and Davies, 1998). This leads to high water use efficiencies under saline conditions, particularly for halophytes (Table 1). The mechanistic basis of the tight control of stomata and its coordination with rates of photosynthesis has not been clearly described. Coarse control of stomata under saline conditions could possibly be achieved through variations in the concentration of the hormone abscisic acid (ABA) transported from the roots (reviewed by Munns *et al.*, 1993). In a series of phylogenetic contrasts mangrove species were found to have higher levels of ABA in their tissues compared to levels in glycophyte sister taxa (Farnsworth and Farrant, 1998). Inherently higher levels of ABA might relate to the generally lower stomatal conductance found in the mangrove species (Ball *et al.*, 1988; Clough and Sim, 1989). Fine control of stomata could be the result of feed-back controls on stomata in response to accumulation of intermediates or products of the Calvin cycle that accumulate because of reduced growth under saline conditions. There is evidence that in addition to K⁺, Cl⁻, and Ca²⁺, sucrose can also be responsible for osmoregulation of stomatal guard cell movements (Talbot and Zeiger, 1998). Experiments comparing stomatal physiology among glycophytes and halophytes have not been performed, thus assessment of what mechanisms are responsible for the often greater control of halophytes over water lost per unit carbon gained, and their resultant high water use efficiencies, compared to glycophytes is not yet understood.

15.3 Minimizing water loss, implications for photosynthetic carbon gain

15.3.1 CONSERVATIVE WATER USE

An unavoidable consequence of photosynthetic carbon gain in higher plants is loss of water through transpiration. Despite growing in environments with an abundance of water (e.g. tidally influenced mangroves and salt marshes), halophytes take up water slowly and have low transpiration rates relative to well-watered glycophytic species (Ball, 1996). Water use declines with increasing salinity. Originally, conservative water use was thought to be important in limiting apoplastic uptake of ions (Scholander *et al.*, 1962; Scholander *et al.*, 1966). However, more recent studies with halophytes, including those under elevated CO₂ (Ball and Munns, 1992; Farnsworth *et al.*, 1996), have shown that salt uptake is not directly coupled with water uptake (Glenn and Brown, 1998). The ion concentrations in the xylem decrease hyperbolically with increase in the volume flux such that the flux of ions to leaves does not increase with the transpiration rate (Ball,

1988), and can even decrease at very high rates of water loss (Munns, 1985; Ball, 1988). Mechanisms of salt exclusion are unknown, but the major means of ion entry into the transpiration stream is via symplastic pathways (Moon *et al.*, 1986; Ball, 1988; Werner and Stelzer, 1990; Lin and Sternberg, 1992), although there is variability among species in the relative importance of symplastic and apoplastic ion uptake (Garcia *et al.*, 1998).

TABLE 1. Water use efficiency of carbon gain expressed as the ratio of photosynthetic carbon assimilation to stomatal conductance (A/g , $\mu\text{mol mol}^{-1}$) in halophytes and glycophytes of different growth forms and photosynthetic pathways grown under varying salinity (in ppt).

Species	Growth form	Salinity	A/g	Reference
Halophytes				
<i>Rhizophora stylosa</i>	tree (C3)	10	67.8	Clough and Sim (1989)
		35	100	
		50	160	
<i>Avicennia marina</i>	"	35	60.8	Sobrado and Ball (1998)
		60	77.5	
<i>Avicennia marina</i>	tree seedling (C3)	3	100	Ball and Farquhar (1984)
		15	125	
		30	128	
<i>Aegiceras corniculatum</i>	"	3	100	Ball and Farquhar (1984)
		15	88.9	
		30	50	
<i>Avicennia germinans</i>	"	0	40.3	Pezeshki <i>et al.</i> (1989)
		20	35.9	
<i>Laguncularia racemosa</i>	"	0	83.6	
		20	67.1	
<i>Rhizophora mangle</i>	"	0	55.7	
		20	59.8	
<i>Atriplex halimus</i>	herb (C3)	0	116	Gale and Pljakoff-Mayer (1970), in Long and Baker (1986)
		9	126	
		27	147	
		39	146	
<i>Scirpus robustus</i>	"	63	144	
		0	58.2	
		15	72	
		30	120	
<i>Salicornia virginica</i>	Succulent herb (C3)	45	71.4	
		0	62.1	
		15	65.6	
		30	71.4	
<i>Salicornia bigelovii</i>	"	45	83.3	Ayala and O'Leary (1995)
		0.5	16.4	
		12	31.3	
		35	47.1	
<i>Spartina foliosa</i>	herb (C4)	0	200	Pearcy and Ustin (1984)
		15	168	
		30	167	
		45	343	
<i>Atriplex prostrata</i> (facultative halophyte)	"	0	44.2	Wang <i>et al.</i> (1997)
		5	74.3	
		10	46.3	

<u>Glycophytes</u>				
<i>Prunus salicina</i>	tree (C3)	3	35	Ziska <i>et al.</i> (1990)
		14	62.5	
		28	63.6	
<i>Fraxinus pennsylvanica</i>	tree seedling (C3)	0	21.5	Pezeshki and Chambers (1986)
		4	20.8	
		8	26.7	
<i>Olea europaea</i>	"	0	53.6	Bongi and Loreto (1989)
		25	73.5	
<i>Taxodium distichum</i>	"	0	138	Pezeshki <i>et al.</i> (1995)
		4	142	
		8	121	
<i>Olea europaea</i> (salt tolerant var. <i>Frantoio</i>)	"	0	65	Tattina <i>et al.</i> (1997)
		10	100	
<i>Taxodium distichum</i>	"	0	43.3	Allen <i>et al.</i> (1997)
		2	42.6	
		4	47.1	
		6	55	
		8	50	
<i>Gossypium hirsutum</i>	herb (C3)	0	50	Brugnoli and Lauteri (1991)
		15	56	
		25	63	
<i>Gossypium hirsutum</i>	"	0	31.8	Brugnoli and Bjorkman (1992)
		9	53.1	
		19	67.9	
<i>Apium graveolens</i> (salt tolerant)	"	0	88	Everard <i>et al.</i> (1994)
		2.5	96	
		10	92	
		30	92	
<i>Triticum aestivum</i>	"	0	45.8	Huang <i>et al.</i> (1994)
		5	55.2	

Several factors may contribute to conservative water use in halophytes. Firstly, low transpiration rates may minimize the accumulation of salt around roots of plants growing in waterlogged soils that are not well flushed by the tides (Passioura *et al.*, 1992). Secondly, it appears that whatever mechanisms are involved in controlling the entry of ions into the transpiration stream may also restrict the flow of water through the roots (Ball, 1988). Thirdly, given a large resistance to water flow in the roots, rapid transpiration rates would induce such a low water potential in leaves that an impossibly high concentration of solutes in cells would be required to maintain turgor (Ball and Passioura, 1993). Finally, rapid induction of very low water potentials would strain the capabilities of the xylem to limit the occurrence and severity of embolism. Indeed, Sperry *et al.* (1988) suggested from their comparative study of water relations in halophytic and glycophytic species of Rhizophoraceae, that mangroves could have evolved to operate with a sufficiently low stomatal conductance to maintain water potentials above a value that is on the threshold of inducing substantial embolism.

In plants that do not have mechanisms to excrete salt arriving in leaves in the transpiration stream, avoiding salt toxicity must be achieved by limiting water loss during photosynthesis through either changes in leaf morphology and plant architecture, and/or through reductions in stomatal conductance. Given the apparent constraints in water use, it is not surprising that leaf level estimates of the water use efficiency of carbon gain, expressed at the ratio of the rate of carbon assimilation (A) over the stomatal conductance (g) are generally higher in halophytes than glycophytes (mean of

A/g for halophytes is 81.7 ± 6.1 and for glycophytes is 52.2 ± 4.7 , t -statistic = 3.78, $P = 0.0004$, Table 1). In glycophytes, A/g generally increases under saline conditions, but A/g is generally lower than what is achieved by halophytes. Woody glycophytes appear to have higher A/g ratios than herbaceous species. Although this trend looks to be similar within the halophytes there is not sufficient data to confirm this. The more conservative water use and higher water use efficiencies indicated by higher A/g ratios in halophytes may reflect the higher carbon costs of water uptake (Ball, 1988) and contribute to maintenance of favorable ion (Ball, 1988) and water relations (Sperry *et al.*, 1988) under saline conditions.

15.3.2 ONTOGENY

The influence of salinity on leaf level measures of water use efficiency in plants can be modified by the ontogenetic stage of the plant (Ziska, 1989; Scartazza *et al.*, 1998). Ziska found in the woody tree species *Prunus salicina* that salinity led to decreases in leaf water potential during fruit expansion, because unlike at other stages in the tree growth, during fruiting stomatal conductance was not reduced by high salinity. Thus, during fruit expansion water use efficiency likely declined (this was not measured directly). Using carbon isotope discrimination techniques, Scartazza *et al.* (1998) also found that the ontogenetic stage, in addition to water deficits, can strongly affect water use efficiency in rice. Plants in their vegetative stage were observed to have lower levels of isotopic discrimination (higher water use efficiencies) when exposed to drought compared to plants that were flowering or filling grain when the drought stress was imposed. These authors suggest that reduced water use efficiency during fruit filling is because 80% of water loss is cuticular and therefore beyond the control of stomata. Whether ontogenetic stage, and particularly reproductive activity alters water use efficiency in halophytes has yet to be determined. Research exploring the sensitivity of halophytes to salinity at different ontogenetic stages may provide insights into the importance of each growth stage to conferring tolerance of salinity.

15.3.3 MORPHOLOGY

High ratios of A/g in halophytes are not only due to reductions in stomatal conductance, but are also due to leaf morphologies that result in the minimization of evaporative demand. Adaptations include steeply inclined leaf angles (e.g. in some mangrove species, Ball *et al.*, 1988; Lovelock and Clough, 1992), and the loss of leaves such that the stems become the major assimilatory organs (e.g. salt marsh species in the genera *Scirpus* and *Salicornia*). Another evolutionary adaptation maybe the adoption of C4 photosynthetic metabolism in some taxa (e.g. *Spartina*, Chapter 16), even though they inhabit cool climates where plants with C3 metabolism are more common (Long, 1983), and the ability to switch from C3 to CAM photosynthetic metabolism (see below and Chapters 16, 17).

Leaf and stem succulence and xeromorphy are also characteristics of halophytes that generally increase as the salinity of the root zone increases (e.g. Lüttge *et al.*, 1989; Smith *et al.*, 1989). Leaf or stem succulence in halophytes was first thought to be a mechanism to dilute salts to avoid toxicity. More recent views are that succulence is due to higher turgor pressure in plants exposed to saline conditions (reviewed by Clough *et*

al., 1982; Long and Baker, 1986; and Ungar, 1991). Glenn and O'Leary (1984) found that succulence increases at moderate salinities in herbaceous halophytes (which was species dependent, ranging between 10 to 30 ppt). But at higher salinities, succulence declines as tissues dehydrate, presumably to concentrate salt and thereby generate sufficiently high osmotic potentials for the maintenance of turgor and growth. In mangrove species that do not excrete salt, leaf succulence increased with ion accumulation in leaves (Popp *et al.*, 1988), and with salinity tolerance of the species (Ball *et al.*, 1988). Those species that excreted salt did not follow this trend (Popp *et al.*, 1988; Smith *et al.*, 1989). Ball *et al.* (1988) suggest that enhanced succulence may enhance the heat capacity of leaves thereby dampening excursions in leaf temperature due to variations in environmental conditions. This may prevent leaf temperatures rising into ranges that are damaging to the photosynthetic apparatus, and/or result in high vapor pressure deficits that drive high rates of transpiration. Leaf 'succulence' may often be confused with leaf thickening (Clough *et al.*, 1982) that can also occur in response to exposure to high levels of sunlight (Ball *et al.*, 1988; Lovelock *et al.*, 1992), leaf age (Smith *et al.*, 1989), and low concentrations of soil phosphorus (Feller, 1996).

Xeromorphy in halophytes is due to thickening of cuticular layers that minimizes evaporation through the cuticle (reviewed by Long and Baker, 1986). Xeromorphy and small average leaf area are adaptations to salinity in many taxa. For example, *Salicornia* has leaves that are reduced to scales (Long and Baker, 1986), *Portulaca rubricaulis* loses its leaves during the dry season when salinities are hypersaline (Lüttge *et al.*, 1989), and the most salt tolerant taxa within the mangrove family Rhizophoraceae have the smallest average leaf area (Ball *et al.*, 1988). Small leaf areas decrease the leaf temperature and hence the vapor pressure deficit under high solar radiation loads by enhancing heat transfer to the surrounding air, as well as increasing the boundary layer conductance to CO₂ diffusion to the leaf (Ball *et al.*, 1988). Both of these factors can contribute to enhanced water use efficiency in halophytes. These adaptations that contribute to enhanced water use efficiency in halophytes do however have considerable costs with respect to growth (see below).

15.3.4 PLANTS THAT SWITCH PHOTOSYNTHETIC PATHWAY

One of the most dramatic photosynthetic responses to salinization has been observed in *Mesembryanthemum crystallinum*, the ice plant (Chapter 16, 17). In this remarkable little plant, salinization of soil results in switching from C3 photosynthetic metabolism to CAM, with a subsequent enhancement in water use efficiency (Winter, 1973; Adams *et al.*, 1998). When a plant has CAM it avoids opening its stomata during the day when evaporative demand is highest, and instead metabolizes stored malate that it has produced at night. At night stomata open and plants produce malate through the activity of the CAM enzyme phosphoenolpyruvate carboxylase, which initially fixes CO₂ to oxaloacetate before it is transformed to malate (Winter and Smith, 1997). Opening stomata during the night when evaporative demand is low and water loss is minimal gives CAM plants an advantage over C3 plants when water availability is limited and temperatures are cool (Winter and Smith, 1997). How commonly this ability to 'switch' occurs within taxa having CAM when plants are exposed to salinity is not known (Winter and Smith, 1997).

The switch from C3 to CAM in ice plants occurs normally during the development of the plant, at 5 weeks of age, but can be accelerated by the imposition of saline conditions (Winter, 1973; Adams *et al.*, 1998). Because of this detectable change in photosynthetic metabolism, the ice plant has been used intensively to study the molecular basis of salt tolerance (Bohnert *et al.*, 1995, see Chapter 17). More recently it has been found that the imposition of salt stress in *Mesembryanthemum crystallinum* enhances the concentrations of low molecular weight carbohydrates (Tarczynski *et al.*, 1993; Shen *et al.*, 1997) and the activity of superoxide dismutase (Miszalski *et al.*, 1998), all of which increase protection from oxidative stress.

15.4 Enhanced risks of photoinhibition and photooxidation

Reduction in photosynthesis under high salinities, caused by either stomatal or non-stomatal limitations, results in an increase in the potential for photooxidative damage to photosystem II, or photoinhibition (Osmond, 1994). Bjorkman *et al.* (1988) were the first to assess photoinhibition in halophytes. They found that plants (in this case mangroves) growing under saline conditions were more photoinhibited than those growing in less saline conditions. Since that time it has been established that photoinhibition can be both an indication of photooxidative damage, and/or regulation of photosystem II, facilitating its protection from photooxidative damage. In the early experiments of Bjorkman *et al.* (1988), photoinhibition in mangrove leaves was reversible indicating this was a regulatory process. The yellow-orange xanthophyll pigments have been shown to be associated with protection of photosystem II from irreversible photoinhibition through a deepoxidation reaction, whereby the inactive violaxanthin is converted to the active zeaxanthin via an intermediate, antheraxanthin (Demmig-Adams and Adams, 1992). Table 2 shows the concentration of xanthophyll pigments, expressed on a unit chlorophyll basis, is generally higher in halophytes compared to glycophytes, however as yet the data set is limited. Within the halophytes increases in salinity do not appear to result in increases in concentrations of xanthophyll pigments (Brugnoli and Bjorkman, 1992; Lovelock and Clough, 1992; Sobrado and Ball, 1998), indicating enhancements of the capacity of photooxidative protection offered by this pathway is not sensitive to salinity, although it is highly sensitive to drought stress (Brugnoli and Bjorkman, 1992).

Protection from photooxidative damage to photosystem II and its components when rates of photosynthetic carbon gain are restricted due to salinity stress can also be achieved by utilizing electrons in other pathways (Powles *et al.*, 1979; Wu *et al.*, 1991; Schreiber and Neubauer, 1990; Loreto *et al.*, 1994; Grace and Osmond, 1995). For example, photorespiration, and the Mehler-peroxidase reactions utilize electron flow from PSII, and have been shown to protect against photooxidative damage (Wu *et al.*, 1991; Lovelock and Winter, 1996). In the Mehler-peroxidase reaction, oxygen directly reacts with electrons to form oxygen radicals that are subsequently neutralized by superoxide dismutase (SOD) to form hydrogen peroxide. Hydrogen peroxide can then be transformed to water in a number of ways (reviewed by Foyer, 1993). The extent of the electron flow to alternative pathways can be expressed as the ratio of electron transport, usually measured via chlorophyll fluorescence, to the actual rate of photo-

TABLE 2. Concentration of xanthophyll pigments (VAZ) expressed on a unit chlorophyll (TChl) basis for halophytes and glycophytes growing in full sunlight.

Species	Salinity (ppt)	VAZ/TChl	Reference
Halophytes			
<i>Avicennia marina</i>	35	195	Sobrado and Ball (1998)
	60	170	
<i>Rhizophora stylosa</i>	9	76	Lovelock and Clough (1992)
	35	148	
<i>Bruguiera parviflora</i>	9	178	
	35	158	
<i>B. gymnorhiza</i>	9	141	
	35	146	
Glycophytes			
<i>Anacardium excelsum</i>	0	88	Koniger <i>et al.</i> (1995)
<i>Cecropia longipes</i>	0	125	
<i>Pseudobombax septenatum</i>	0	81	Brugnoli and Bjorkman (1992)
<i>Gossypium hirsutum</i>	0	123	
	9	152	
	19	156	
<i>Euonymus kiutchovicus</i>	0	119	Demmig-Adams and Adams (1992)
<i>Mahonia repens</i>	0	113	
<i>Parthenocissus quinquefolia</i>	0	115	
<i>Vinca minor</i>	0	88	

synthetic carbon gain. Theoretically 4 electrons are required to fix one molecule of CO_2 . C4 species, which concentrate CO_2 in their bundle sheathes, approach this theoretical minimum (see *Panicum maximum*, Table 3), while C3 plants have ratios greater than 4, except when they are measured in the absence of oxygen or at extremely high CO_2 concentrations. If the ratio is more than 4 then other processes are consuming electrons. Although the data are limited, it appears that halophytes may have an enhanced capacity for flow of electrons to alternative pathways, the capacity of which may increase with increasing salinity (Table 3). Importantly Cheeseman *et al.* (1997) also found that the activity of SOD was forty times greater in the mangrove *Rhizophora stylosa* growing in the field in North Western Australia than the activity of SOD in peas. They also observed that the activity of SOD was lower in *R. mangle* growing in fresh water in a greenhouse in Illinois, but that even in these greenhouse grown mangroves SOD activities were still 5 times greater than those found in peas. Interestingly the components of the Mehler-peroxidase reaction responsible for detoxifying the product of SOD, hydrogen peroxide, had similar activities in all plants. This suggests that mangroves and other halophytes may have novel ways of removing high concentrations of hydrogen peroxide, perhaps through the activities of phenolics or peroxidases (Cheeseman *et al.*, 1997), or through the production of oxygen radical scavenging low molecular weight carbohydrates (Tarczynski *et al.*, 1993; Shen *et al.*, 1997). When exposed to saline conditions the concentration of soluble peroxidases was enhanced in the halophyte *Atriplex prostrata* (Wang *et al.*, 1997), while in mangroves increases in salinity have lead to increases in concentrations of mannitol, proline and other low molecular weight carbohydrates (Popp, 1984; Richter *et al.*, 1990).

There is very little data on photoinhibition and photoprotection in halophytes. Our preliminary conclusions, from the available data, suggest that photoprotective processes

TABLE 3. Ratio of rate of electron transport (ETR) through photosystem II and rate of photosynthetic carbon gain (A) in both halophytes and glycophytes.

Species	Salinity (ppt)	ETR/A ratio	Reference
Halophytes			
<i>Avicennia marina</i>	35	9.3	Sobrado and Ball (1998)
	60	18.3	
<i>Rhizophora stylosa</i>	35	22.9	Cheeseman <i>et al.</i> (1997)
Glycophytes			
<i>Ficus insipida</i>	0	6.2	Lovelock and Winter (1996)
<i>Luehea seemannii</i>	0	9.1	
<i>Psuedobombax septenatum</i>	0	16.7	
<i>Didymopanax morototoni</i>	0	6.5	
wheat	0	9.8	Krall and Edwards (1992)
<i>Flaveria pringlei</i>	0	12.1	
<i>Panicum maximum</i> (C4)	0	4.6	Hausler <i>et al.</i> (1994)
barley	0	8.5	

are enhanced in halophytes compared to glycophytes and this may be important in tolerance of salinity, particularly if salinity is experienced in conjunction with other stressors (e.g. high levels of solar radiation or drought). More data needs to be collected to assess this hypothesis.

15.5 Relationship between photosynthetic carbon gain and growth under saline conditions.

Reductions in growth under saline conditions can be due to changes in biomass allocation patterns (e.g. allocation of biomass to root and stem tissue rather than to leaves that can assimilate more carbon), or due to increases in respiration, or reductions in photosynthetic carbon gain (Munns, 1993). Growth analysis techniques allow researchers to determine which of these factors causes reductions in growth rates. Relative growth rate is determined by the leaf area available for carbon assimilation, expressed as the leaf area per plant biomass (leaf area ratio, LAR) and the net assimilation rate (NAR) of carbon per unit of area. The NAR is dependent on the rate of photosynthetic carbon gain and the rate of respiration. In the few studies that have analyzed the growth of plants over a wide range of salinities (De Jong, 1978; Longstreth *et al.*, 1984; Percy and Ustin, 1984; Jansen *et al.*, 1986; Long and Baker, 1986; Ball, 1988; Ball and Pidsley, 1985) it has generally been found that there are important and large interspecific differences in the relative contributions reductions in LAR and NAR play in reducing growth rates under high salinity (Table 4). However in most cases, reductions in NAR play a significant role in reducing relative growth rates when salinity of the rooting medium is increased (Figure 1). Because respiration rates are not greatly altered by salinity (Long and Baker, 1986), much of the reductions in growth due to increased levels of salinity are due to reductions in photosynthesis.

Both succulence and xeromorphy reduce the ratio of leaf area to total biomass of the plant (LAR) and thereby lead to decreased relative growth rates (Figure 1), if not matched by enhanced rates of carbon assimilation. Rawson (1986) stated that by only

TABLE 4. Proportional reduction in relative growth rate (RGR), leaf area ratio (LAR) and net assimilation rate (NAR), or rate of photosynthetic carbon gain (*) in studies where plants were grown over a range of salinity (in ppt).

Species	Salinity	RGR	LAR	NAR	Reference
<i>Alternanthera philoxeroides</i>	0	1	1	1	Longstreth <i>et al.</i> (1984)
	6	0.91	0.68	0.88	
	12	0.59	0.45	0.80	
	18	0.36	0.23	0.54	
	24	0.18	0.23	0.50	
<i>Avicennia marina</i>	3	1	0.96	1	Ball (1988)
	15	0.67	1	0.90	
	30	0.59	0.96	0.79	
<i>Aegiceras corniculatum</i>	3	1	0.89	1	Pearcy and Ustin (1984)*
	15	0.51	1	0.69	
	30	0.28	0.71	0.31	
<i>Spartina foliosa</i>	0	0.94	1	0.95	
	15	1	0.96	1	
	30	0.67	0.81	0.98	
<i>Scirpus robustus</i>	45	0		0.57	Ball and Pidsley (1995)
	0	1	0.86	0.89	
	15	0.69	1	1	
	30	0.35	0.47	0.5	
	45	0.23	0.29	0.14	
<i>Salicornia virginica</i>	0	0.9	0.5	0.92	Long and Baker (1986)
	15	1	1	0.84	
	30	0.9	0.76	0.80	
	45	0.75	0.76	1	
	0	0.58	0.77	0.58	
<i>Sonneratia alba</i>	2	1	1	0.79	Ball and Pidsley (1995)
	9	1	0.88	1	
	17	1	0.84	0.94	
	26	0.81	0.84	0.79	
	35	0.63	0.85	0.48	
<i>Sonneratia lanceolata</i>	0	0.98	0.60	1	Long and Baker (1986)
	2	1	0.63	0.99	
	9	0.76	0.67	0.71	
	17	0.51	1	0.37	
<i>Aster tripolium</i>	0	1	1	0.96	Long and Baker (1986)
	10	0.89	0.86	1	
	20	0.58	0.76	0.73	
	30	-0.05	0.46	-0.12	
<i>Atriplex californica</i>	0	0.73	0.76	0.88	Long and Baker (1986)
	9	0.95	0.88	1	
	18	1	0.96	0.96	
	36	0.25	1	0.23	
<i>Ambronía marítima</i>	0	0.36	0.81	0.44	Long and Baker (1986)
	9	1	1	1	
	18	0.83	0.89	0.93	
	36	0.41	0.92	0.44	
<i>Oryza sativa</i>	0	1	1	1	Long and Baker (1986)
	1.5	0.91	0.91	1	
	3	0.78	0.8	0.97	
	5	0.32	0.7	0.46	

considering measures of gas exchange without considering carbon allocation patterns researchers could come to misleading conclusions about salinity tolerance. This is exemplified by the halophyte *Salicornia bigelovii*. Rates of photosynthesis of *Salicornia bigelovii* were maintained at approximately $17 \mu\text{mol m}^{-2} \text{s}^{-1}$ when salinity of the substrate increased from 12 to 35 ppt, however the size of the plants growing at 35 ppt was approximately half that of plants growing at 12 ppt after 40 days of growth (Ayala and O'Leary, 1995). Similarly, under highly saline conditions, in the more salt tolerant of two mangrove species, *Avicennia marina*, the ratio of leaf area to total plant biomass was reduced more than in the less salt tolerant species, *Aegiceras corniculatum* (Ball, 1988). In contrast, a study of two mangrove congenitors, the ability of *Sonneratia alba* to maintain growth at higher salinities compared to the less salt tolerant *S. lanceolata*, was associated with the maintenance of higher rates of net assimilation (Ball and Pidsley, 1995). Therefore, tolerance of salinity is a whole plant phenomenon, requiring complex integration of carbon gain in relation to water loss and ion uptake at all levels of plant function. Understanding mechanisms by which such integration is achieved remains a major challenge.

15.6 Future Directions

15.6.1 EUTROPHICATION AND PHOTOSYNTHESIS OF HALOPHYTES

Eutrophication is a major influence in many coastal regions. The addition of nutrients usually causes an increase in the capacity for photosynthetic carbon gain (Evans, 1989). In agricultural systems, fertilization generally increases the salt tolerance of crops, particularly if the salinity stress is moderate (Hu *et al.*, 1997 and references therein), but the benefits to yield of fertilization are often lost at high levels of soil salinity. There have been few studies of the effects of nutrient enrichment on salinity tolerance of halophytes in naturally saline ecosystems.

It is known that plants within saline ecosystems are often nutrient limited, and increases in nutrient availability results in enhanced plant growth rates (e.g. Smart and Barko 1980; Naidoo, 1987; Feller, 1995; Kock and Snedaker, 1998) and/or plant stature (Valiela and Teal, 1974; Boto and Wellington, 1984; Kao and Chang, 1998). In laboratory experiments with the salt marsh species, *Spartina alterniflora*, increasing salinity led to an increase in level of tissue nitrogen critical for growth (Bradley and Morris 1992). However, in a fertilization study of salt marshes dominated by *Spartina alterniflora*, fertilization led to an increase in leaf nitrogen concentrations, but enhancements in rates of photosynthesis were not observed, possibly because of self shading of leaf surfaces within the dense canopy (Dai and Wiegert, 1997). Increased nutrient availability in a laboratory study has led to increases in photosynthetic rates in the mangrove, *Rhizophora mangle*, growing under saline conditions (Lin and Sternberg, 1992).

Fertilization under saline conditions can lead to higher water use efficiency of carbon gain. In the study of the mangrove *Rhizophora mangle*, fertilization significantly improved water use efficiency (A/g) at salinities up to 50 ppt (Lin and Sternberg, 1992).

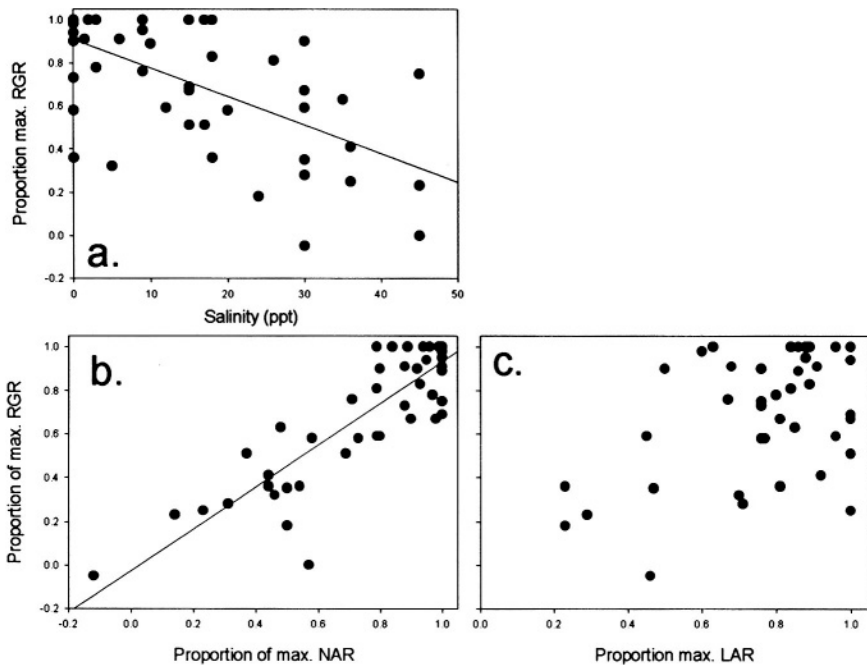


Figure 1. Proportional response of relative growth rate (RGR) to varying salinity (a), and the contributions of net assimilation rate (NAR, b) and leaf area ratio (LAR, c) to relative growth rate. Data is from studies listed in Table 4.

The enhancement in water use efficiency was no longer evident when trees were exposed to an additional stress, the presence of sulphide, which is common in anaerobic, waterlogged soils. Enhanced water use efficiency in fertilized plants in this experiment occurred because of a greater relative increase in photosynthetic carbon gain compared to stomatal conductance. Similar results have been found when trees of another mangrove species, *Avicennia germinans*, were fertilized when growing in hypersaline conditions in the field (C.E. Lovelock and I.C. Feller, unpublished data).

Enhanced water use efficiency in plants has been correlated with decreased nitrogen use efficiency, or the photosynthetic rate per unit nitrogen, both among (Field *et al.*, 1983) and within species (Reich *et al.*, 1989; Wang *et al.*, 1998). It was proposed that there is a trade-off between water use efficiency and nitrogen use efficiency (Field *et al.*, 1983), and that plants maximize the utilization efficiency of the most limiting resource (Reich *et al.*, 1989). These ideas have not yet been thoroughly tested in halophytes. At a hypersaline site in Florida, the more salt tolerant mangrove species, *Avicennia germinans*, had higher water use efficiency and lower nitrogen use efficiency than that of the less salt tolerant species *Laguncularia racemosa* (C.E. Lovelock and I.C. Feller, unpublished data). This suggests a trade-off between nutrient use efficiency and water use efficiency among species of mangroves that may be linked to their relative salinity tolerance. The mechanistic basis underlying the compromise between water use efficiency and nitrogen use efficiency under saline conditions is not clear. The type of

compound used for osmoregulation may be key to the compromise. In this example, *A. germinans* uses the nitrogen rich compound glycine betaine for osmoregulation, while in *L. racemosa*, mannitol is in high concentrations (Popp and Polania, 1989). Another possible way in which water use efficiency could be influenced by fertilization is through fertilized plants increasing the salinity of the substrate. Higher rates of photosynthesis and thus water loss because of fertilization could result in greater rates of salinization as salt is drawn to the root surface of the transpiring plant (Chalmers, 1979; Smart and Barko, 1980; Passioura *et al.*, 1992). In the experiment in the Florida mangroves, the salinity of soil under fertilized trees was higher than those under unfertilized trees (C.E. Lovelock and I.C. Feller, unpublished data).

In some species under saline conditions nitrogen may be reallocated to enzymes and molecules that may enhance salinity tolerance, without increasing rates of photosynthesis. For example, nitrogen may be allocated toward processes that protect against photooxidative damage (e.g. enzymes of the xanthophyll cycle, Verhoeven *et al.* 1996, or those involved in the Mehler peroxidase reactions), and also toward the production of nitrogen containing solutes required for osmotic adjustment of the cytosol (Popp *et al.* 1984, Chapters 9 and 11). Diversion of nitrogen to protective functions would increase the apparent requirement for nitrogen and also decrease the nitrogen use efficiency. Reallocation of nitrogen under saline conditions may explain observations of decreases in rates of photosynthesis during conditions of high salinity and drought despite a constant nitrogen concentration in leaves of *Avicennia germinans* growing under field conditions (Sobrado, 1999). An additional complexity in predicting the influence of nutrient availability on salinity tolerance in the field may arise because species composition can not only influence salinity of substrates but also may influence soil microbial processes and nutrient availability (Sherman *et al.*, 1998 for mangrove forests).

There is not enough evidence to clearly understand the consequences of nutrient additions in saline environments. In most situations nutrient additions are likely to cause increases in water use efficiency of plants. If, however, salinization of the substrate due to enhanced rates of transpiration constrains stomatal conductance and photosynthesis in some species and not others, the ecological consequences of nutrient enrichments in saline environments could be complex. Species that have higher tolerances of salinity, and/or utilize nitrogen rich osmotically active solutes, may thrive under nutrient enriched conditions at the expense of less tolerant species.

15.6.2 EFFECTS OF SALINITY ON PHOTOSYNTHESIS UNDER GLOBAL CLIMATE CHANGE

15.6.2.1 Elevated CO₂

The concentration of CO₂ in the atmosphere is steadily rising and is expected to reach approximately 700 ppm in this century. Because elevated CO₂ usually leads to declines in stomatal conductance, increases in photosynthesis and improvements in water use efficiency (reviewed in Drake *et al.*, 1997), the continuing rise of atmospheric CO₂ concentrations may favor photosynthesis and growth in halophytes under saline conditions where much of the reduction in growth can be due to limitations to photosynthetic carbon assimilation (see above). Ball and Munns (1992) reviewed the

scarce literature available that addresses the response of halophytes to variations in salinity under elevated CO_2 . They found that growth and photosynthesis were stimulated by elevated CO_2 when plants were grown under moderate salt stress, more than at conditions optimal for growth. Thus, when stomatal closure due to high salinity limits carbon gain, the increase in intercellular CO_2 under elevated CO_2 conditions ameliorates the effects of stomatal closure. More recently elevated CO_2 was found not to result in increased growth or net assimilation rates when mangroves were grown at supraoptimal salinities (Ball *et al.*, 1997). Similarly, only a small increase in photosynthetic rate was observed in *Rhizophora mangle* grown at salinities equivalent to seawater, and this enhancement only occurred early in the experiment (Farnsworth *et al.*, 1996). The failure of elevated levels of CO_2 to increase growth and photosynthesis in high salinities (but salinities that are normal for these plants) indicates that factors other than carbon were limiting growth in these experiments. Possibly under high salinities photosynthesis and growth are more limited by nutrient deficiencies (e.g. salinity induced potassium deficiencies) or salt toxicity than by the availability of carbon (Ball and Munns, 1992).

In saline conditions water use efficiency is generally enhanced under elevated compared to ambient CO_2 (reviewed in Ball and Munns, 1992). More recent studies confirm this finding on both the leaf level (Farnsworth *et al.*, 1996; McKee *et al.*, in press) and for whole plant growth (Ball *et al.*, 1997), although, in less salt tolerant species at high salinities this effect was small and not statistically significant (Ball *et al.*, 1997). Therefore, it appears that elevated levels of CO_2 will not greatly affect growth, photosynthesis or water use efficiency where salinity limits growth. Instead increases in productivity under elevated CO_2 are likely under moderate salinities where plants have a substantial potential for growth, perhaps enhancing differences in productivity already apparent between high and moderate salinity sites (Ball *et al.*, 1997). There is certainly no evidence that elevated CO_2 will increase the ecological amplitude of halophytes such they can invade sites where extreme salinities preclude growth.

In saline environments transpiration of plants can have an effect on the salinity of the soil (Chalmers *et al.*, 1979; Passioura *et al.*, 1992). This is because as plants draw water to the root surface salts are concentrated within the soil. Flushing by tidal flow rarely ameliorates this process because soils are often poorly drained and diffusion of salts is slow. If elevated CO_2 does decrease water use by plants then one possible consequence is that soils could have lower salinities. In the experiments of Ball *et al.* (1997) whole shoot water use varied unpredictably with exposure to elevated CO_2 . In salt marshes exposed to elevated CO_2 whole canopy transpiration was reduced (Drake *et al.*, 1992). Additionally, whether salinity of substrates will be affected by reduced water use of plants under elevated CO_2 will largely depend on whether leaf level reductions in stomatal conductance are translated into reduced whole canopy water loss. In large trees, stomatal conductance was poorly correlated with whole branch transpiration flow (Meinzer *et al.*, 1997; Andrade *et al.*, 1998). Thus, the ability to predict changes in ecological patterns within halophytic vegetation as CO_2 concentrations rise could be further complicated by alterations in soil water availability brought about by stomatal responses to elevated CO_2 .

15.6.2.2 *Rising sea-level*

Few studies have examined the possible effects of rising sea-levels (flooding) on photosynthetic carbon gain in halophytes. There is however, a growing literature assessing the influence of salt water incursions into coastal wetlands, a scenario that becomes increasing likely with rises in sea level and increased frequency of severe storms. In the mangrove *R. mangle*, maximum rates of photosynthetic carbon gain and stomatal conductance were reduced when plants were exposed to flooding regimes that simulated a 16 cm increase in tidal height (Ellison and Farnsworth, 1999). Within mangrove taxa there are species differences in response to flooding (McKee, 1997). However, in a study of three mangrove species there was no interaction between the effects of salinity and flooding on photosynthesis or growth (Pezeshki *et al.*, 1989). Presently there is insufficient data to assess how increases in sea level will affect salt tolerance of halophytic vegetation.

In studies of glycophytes exposed to increases in salinity to simulate saltwater incursions in coastal areas, the effects of salinity are generally exacerbated when plants are flooded when the species studied are ones that are not normally flooded. In a study examining the effects of salinity on flooding tolerance in cabbage palm both seedling survival and rates of photosynthetic carbon gain were depressed to a greater extent when plants were inundated at high salinities than when they were flooded at lower salinities (Perry and Williams, 1996). Salinity in the range of 3-8 ppt decreased photosynthetic rates and increased mortality of green ash (Pezeshki and Chambers, 1986), the herb *Sagittaria lancifolia* (Pezeshki *et al.*, 1987), baldcypress (Allen *et al.*, 1997), and a range of oak species (Connor *et al.* 1998), indicating that seawater incursions into coastal vegetation are likely to be disastrous. In a comparative study of four coastal tree species responses to short term flooding differed among species indicating exposure to salinity during storm surges could cause changes in the species composition of coastal vegetation.

15.7 Conclusions

Under low salinity conditions, increasing salt tolerance of species occurs at the expense of growth and competitive ability, but the conservative growth strategies of plant species that are tolerant of high salinities lead to competitive advantages under highly saline conditions (Ball, 1996). Within this unavoidable compromise, maintenance of photosynthetic processes under saline conditions is key to growth and productivity. Research that is enhancing our understanding of the mechanisms of salinity tolerance of photosynthetic processes are in the areas of the importance of anti-oxidants (e.g. Cheeseman *et al.*, 1997), including the new consideration of the role of low molecular weight carbohydrates (e.g. Tarczynski *et al.*, 1993; Shen *et al.*, 1997; Gilbert *et al.*, 1997). Additionally the investigation of the causes and controls of mesophyll resistance to CO₂ diffusion, and the regulation of salt uptake and water loss are also providing new understanding of how morphological plasticity and physiology interact to influence salinity tolerance.

Areas of research that may prove rich in facilitating our understanding of salinity tolerance are comparative studies of stomatal physiology among glycophytes and

halophytes. This may help illuminate what mechanisms might be responsible for the greater control of halophytes over water lost per unit carbon gained, and their resultant higher water use efficiencies compared to glycophytes. Research exploring the sensitivity of halophytes to salinity at different ontogenetic stages may also provide insights into factors that confer tolerance of salinity (see also Chapter 14).

Given the current rates of eutrophication of coastal zones throughout the world increasing our understanding of how halophytic plant communities will be altered by nutrient additions is extremely important. Nutrient effects on photosynthesis and water loss under saline conditions are likely to be key processes in determining the outcome of eutrophication and therefore deserve high research priority. Other anthropogenic induced environmental changes also deserve more careful examination. Although increases in atmospheric CO_2 are unlikely to alter ecological amplitude of species with respect to salinity (Ball and Munns, 1992), other changes, in productivity, tissue quality and water uptake may certainly have a large impact on halophytic vegetation. Finally, there is insufficient data to assess how increases in sea level will affect salt tolerance of halophytic vegetation.

15.8 References

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CHAPTER 16

PERFORMANCE OF PLANTS WITH C_4 -CARBOXYLATION MODES OF PHOTOSYNTHESIS UNDER SALINITY

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Abstract

The C_4 -carboxylation mode of photosynthetic CO_2 -fixation expressed in C_4 - and CAM-plants provides ecophysiological mechanisms for tolerance of drought stress and hence osmotic stress. Therefore, one would also expect occurrence of a large diversity of C_4 - and CAM-plants among halophytes. This, however, is not the case. Halophytes among C_4 -plants are various grasses and species of *Atriplex* tolerating salinity by salt extrusion via salt glands and bladders, respectively. CAM-performing cacti in saline habitats are salt excluders, i.e. stress avoiders. Only the Aizoaceae *Mesembryanthemum crystallinum*, where high salinity enhances a shift from C_3 -photosynthesis to CAM, is a truly salt-resistant salt-including CAM-plant.

16.1 CAM plants and C_4 -plants among halophytes

C_4 -carboxylation modes of photosynthesis use phosphoenolpyruvate carboxylase (PEPC) for primary fixation of CO_2 . The first stable CO_2 -fixation products are organic acids, mainly malic acid (or malate at cytoplasmic pH). This provides internal CO_2 -concentrating effects. In plants with Crassulacean acid metabolism (CAM; see also Chapter 17) primary CO_2 -fixation occurs in the dark period, malic acid is stored in the vacuole and malate remobilization and decarboxylation during the light period lead to high internal CO_2 concentrations for refixation via ribulose-bis-phosphate carboxylase/oxygenase (RUBISCO) and assimilation in the Calvin cycle behind closed stomata (Cockburn *et al.*, 1979; Kluge *et al.*, 1981). In C_4 -plants primary CO_2 -fixation occurs in the light in peripheral mesophyll tissue, concomitantly malate is transported to more central bundle sheath tissue where decarboxylation also causes high CO_2 -concentrations, i.e. about 6 to 10 times higher than in the ambient atmosphere (Hatch, 1987), behind a CO_2 -impermeable suberized barrier in the apoplast. With these CO_2 -concentrating effects the two C_4 -carboxylation modes are modifications of photosynthesis supporting operation under stress of limited availability of water with

totally or partially closed stomata during periods of the strongest insolation of the day. This leads to an improved water-use-efficiency (WUE: mol CO_2 fixed per mol H_2O transpired) as compared to **C₃-plants**. Salinity *per se* or often connected with drought is one of the major known causes of water stress to plants. Should one therefore not expect a large diversity of CAM and **C₄-plants** among halophytes? What does the literature provide on this point?

Among constitutive halophytes the mangrove *Rhizophora mangle* was shown to produce day/night changes of malate levels of 36 mM when grown at 200 mM NaCl as compared to 15 mM in a salt free medium (Werner and Stelzer, 1990). If significant at all, this is minute in comparison to the up to more than 10 times larger nocturnal malate accumulation achieved by dark **CO₂-fixation** in *bona fide* CAM plants.

Although in leafy cacti in the Pereskioideae and Opuntioideae CAM-capacity is weak in the leaves, in the succulent cladodes of Opuntioideae and stems of Ceroideae among the cacti CAM is always constitutive and obligatory (Nobel and Hartsock, 1987). A survey of ion levels in several cacti in relation to nocturnal acid accumulation and growth has shown that both were inhibited by Na^+ in the chlorenchyma (Nobel, 1983). Although there was a certain partitioning of Na^+ arriving in the shoots, with higher Na^+ concentrations in the central water storage parenchyma than in peripheral chlorenchyma, salt sensitivity of the cacti was high; 100 mM NaCl in the root medium already caused 50 % growth inhibition. Individuals of coastal populations of the cactus *Opuntia humifusa* accumulated more Na^+ in their cladodes and appeared to be better adapted to aerial salt spray as well as episodal high salinity in their root medium than inland individuals (Silverman *et al.*, 1988; see below Figure 3). Among the Macaronesian Sempervivoideae in the Crassulaceae there are CAM plants which may be adapted to saline conditions, e.g. *Aeonium sedifolium* and other members of this genus (Lösch and Kappen, 1981). *Agaves* are also obligate CAM plants. Even the desert species *Agave deserti*, however, is quite salt sensitive (Nobel and Berry, 1985).

For **C₄-photosynthesis** among halophytes *Salsola soda* is a **C₄-plant** (Karabourniotis *et al.*, 1983), *Suaeda monoica* was considered to be a **C₄-plant** without typical bundle sheaths (Shomer-Ilan *et al.*, 1975). Many **C₄-grasses** are more or less halotolerant (Table 1). The grass *Aeluropus littoralis* is **C₃/C₄** intermediate (Shomer-Ilan and Waisel, 1973, 1976). The **C₄-cereal** *Sorghum bicolor* has cultivars which can be adapted to growth under salinity (Amzallag, 1990 a; Chapter 14). For **C₄-photosynthesis** it is also appropriate to look around the „salt bushes“, i.e. *Atriplex* species in the Chenopodiaceae, which can excrete NaCl by accumulation in epidermal bladders and normally are quite salt resistant. Many of them are **C₄-plants** (Slatyer, 1970). With one exception all endemic *Atriplex* species of Australia have **C₄-photosynthesis** (Osmond, 1974).

In the **C₄-species** *Atriplex lentiformis* salinity decreases the ratio of **C₃-cycle** activity (RUBISCO) to **C₄-cycle** activity (PEPC) by reduced partitioning of RUBISCO-activity relative to PEPC-activity. This helps maintaining high water-use efficiency while reducing light-use efficiency due to the higher energy requirement of **C₄-photosynthesis**, but the latter may not be limiting at the exposed sites occupied by **C₄-plants**. A more severe constraint for *this Atriplex* species under salt stress may be that

TABLE 1. Halophytic C₄-grass genera

<i>Aeluropus</i>	halophytic
<i>Alexfloydia</i>	halophytic to glycophytic
<i>Cenchrus</i>	halophytic or glycophytic
<i>Chaetobromus</i>	halophytic or glycophytic
<i>Cladoraphis</i>	halophytic or glycophytic
<i>Coelachyrum</i>	halophytic or glycophytic
<i>Crypsis</i>	halophytic or glycophytic
<i>Cynodon</i>	halophytic or glycophytic
<i>Dactyloctenium</i>	halophytic or glycophytic
<i>Daknopholis</i>	halophytic (maritime sand, Madag.)
<i>Dichanthium</i>	halophytic or glycophytic
<i>Diplachne</i>	halophytic or glycophytic
<i>Distichlis</i>	halophytic
<i>Entoplocamia</i>	halophytic or (most) glycophytic
<i>Eragrostis</i> (350 species)	halophytic or glycophytic
<i>Eulalia</i>	halophytic or glycophytic
<i>Halopyrum</i> (1 species)	halophytic
<i>Heterocarpha</i> (1 species)	halophytic
<i>Imperata</i>	halophytic or glycophytic
<i>Ischaemum</i>	halophytic or glycophytic
<i>Jouvea</i>	halophytic or glycophytic
<i>Leptochloa</i>	halophytic or glycophytic
<i>Leptochloopsis</i>	halophytic or glycophytic
<i>Lepturidium</i> (1 species)	halophytic
<i>Lepturopetium</i>	halophytic (?)
<i>Lepturus</i>	halophytic
<i>Monanthochloe</i> (3 species)	halophytic
<i>Myriostachya</i> (1 species)	halophytic
<i>Neostapfiella</i> (3 species)	halophytic or glycophytic
<i>Odyssea</i> (2 species)	halophytic
<i>Oxchlois</i> (1 species)	halophytic to glycophytic
<i>Panicum</i> (C ₃ or C ₄) (370 species)	halophytic and glycophytic (most)
<i>Parectenium</i> (1 species)	halophytic to glycophytic
<i>Pascopyrum</i> (1 species)	halophytic
<i>Paspalum</i> (320 species)	seldom halophytic: e.g. <i>P. distichum</i>
<i>Polypogon</i>	halophytic or glycophytic
<i>Porteresia</i> (1 species)	halophytic
<i>Pseudozoysia</i> (1 species)	halophytic
<i>Psilolemma</i> (1 species)	halophytic
<i>Reederochloa</i> (1 species)	halophytic
<i>Schizachyrium</i> (60 species)	a few species halophytic
<i>Sclerodactylon</i> (1 species)	halophytic
<i>Spartina</i>	halophytic
<i>Sphenopus</i>	halophytic
<i>Spinifex</i>	halophytic
<i>Sporobolus</i> (160 species)	halophytic or glycophytic
<i>Stenotaphrum</i>	halophytic or glycophytic
<i>Thuarea</i>	halophytic
<i>Trachys</i>	halophytic or glycophytic
<i>Uniola</i>	halophytic
<i>Urochondra</i>	halophytic
<i>Willkommia</i> (2 species)	halophytic
<i>Zoysia</i>	halophytic

By courtesy of Hubert Ziegler, München, Germany

salinity diminishes the inherent efficiency of the **CO₂-concentrating** mechanism of **C₄-photosynthesis** by increasing bundle sheath leakiness for CO₂ (Zhu and Meinzer, 1999).

An intriguing question, which arose in relation to **Na⁺** nutrition, is whether **Na⁺** is at all an essential element in plants as it is so well established, of course, for animals. Although halophytes can cope with large amounts of NaCl, essential requirement of **Na⁺** in plants was often negated. Early studies put forward the conclusion that **Na⁺** is required for **C₄-photosynthesis** (Brownell and Crossland, 1972), which was critically checked repeatedly (Ohta *et al.*, 1987; Matoh and Murata, 1990; Brownell and Bielig, 1996) so that it now appears to be well established that at least in a certain group of **C₄-plants** („Na-type **C₄-species**“) **Na⁺** is required for light-dependent active **pyruvate/Na⁺-cotransport** into the chloroplasts of the mesophyll tissue for **C₄-carboxylation** (Ohnishi and Kanai, 1987; Ohnishi *et al.*, 1990; Aoki and Kanai, 1997). An early report also claimed that the CAM-plant *Kalanchoe tubiflora* needs **Na⁺** for CAM but not when performing **C₃-photosynthesis** (Brownell and Crossland, 1974). However, subsequently the literature remained silent about this effect.

This is the meager yield of actual observations in contrast to the expected large diversity of relations of CAM and **C₄-photosynthesis** to salinity. There is little **C₄-photosynthesis** among halophytes and in general CAM plants are not halophytic and halophytes are not CAM plants (Lüttge and Smith, 1984). This apparent paradox also would constitute the end of this review if it were not for two serendipities related to Californian nostalgies.

16.2 CAM and salinity serendipities

Tired one day of studying the tough literature and thus browsing in the library of UC Riverside I found a beautiful photograph of columnar cacti of *Cerus validus* in the extremely saline Salinas Grandes of Argentina (Yensen *et al.*, 1981). Alas, there it was, the CAM-halophyte.

More than ten years earlier having studied the energy dependent NaCl accumulation against a high concentration gradient in the epidermal bladder cells of the halophytic **C₄-Chenopodiaceae** *Atriplex spongiosa* in Australia (Osmond *et al.*, 1969; Lüttge and Osmond, 1970; Lüttge *et al.*, 1970) and reporting this to Andy Benson in La Jolla, California, he rushed out to the coast in excitement and provided the Aizoaceae *Mesembryanthemum crystallinum*, the common ice plant, with its much larger epidermal bladder cells (Haberlandt, 1904) to study salt accumulation there. Seeds were carried home, but it turned out that while the *Atriplex* bladders have a salt gland-like stalk cell and actively excrete NaCl into the bladder vacuole (refs. cited above), the *Mesembryanthemum* bladders are simply inflated epidermal cells without associated gland activity, and although they can store large amounts of water and salt they do not concentrate the latter above the levels found in the mesophyll (Figure 1; Lüttge *et al.*, 1978). Untidily the left-over plants of this study with the labels giving their NaCl

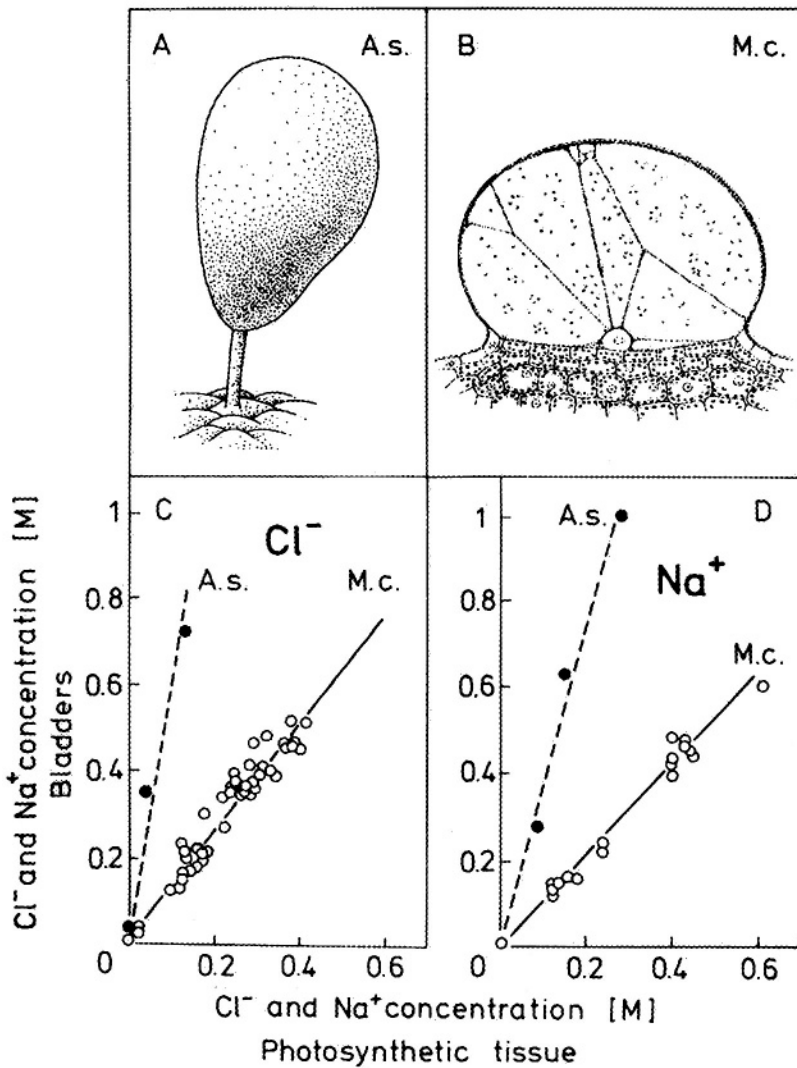


Figure 1. Epidermal bladder with glandular stalk cell of *Atriplex spongiosa* (A) and epidermal bladder cell of *Mesembryanthemum crystallinum* (B; from Haberlandt, 1904) and the relations of Cl^- (C) and Na^+ concentrations (D) in bladders and green photosynthetic leaf cells of both species grown at different NaCl-concentrations in the root medium (after Lüttge *et al.*, 1978).

treatments remained in the green-house, and acquiring them in the absence of a better choice for gas exchange exercises in his class-experiments the then student Klaus Winter discovered the induction of CAM by salinity in *M. crystallinum* (Winter and von Willert, 1972). Checking now 25 years later the large number of entries of the key

word "salinity" in the subject index of the latest book on CAM (Winter and Smith, 1996 a) one finds that they exclusively refer to *M. crystallinum*. Thus, this anecdotal review of CAM and salinity presents us with two case stories of the greatly different life forms of the perennial columnar cacti and the annual ice plant to evaluate the anatomical, morphological and developmental relations of CAM and salinity (Lüttge, 1993).

16.3 Case stories of two different life forms of CAM plants and salinity

16.3.1 STRESS AVOIDANCE *VERSUS* STRESS TOLERANCE

In tropical coastal salinas of the alluvial sand plains at the northern Caribbean coast of Venezuela, where we studied halophytes, there were CAM plants with total stress avoidance such as the epiphytes *Tillandsia recurvata* (Bromeliaceae) and *Schomburgkia humboldtiana* (Orchidaceae) and the terrestrial tank-root type bromeliad *Bromelia humilis* which was only loosely lying on the ground and at the most had very minimal root contact with the soil (Griffiths *et al.*, 1989; Lee *et al.*, 1989). The creeping fruticose C_4 -species *Portulaca rubricaulis* shed its leaves during the dry season when a salt crust was formed on the sand plain (Lüttge *et al.*, 1989 b). Such avoidance strategies are trivial and shall not be further considered here.

Another salinity-stress avoidance strategy is salt exclusion at the root level as in contrast to the tolerance strategy of salt inclusion. The salt-includer/salt-excluder conception has played a large role in evaluation of salinity adaptations although it is clear that exclusion/inclusion is never absolute and can only be considered in relative terms. Nevertheless, our comparison of perennial cacti and the annual *M. crystallinum* allows us to illustrate the distinction quite well (Figure 2). *M. crystallinum* is a salt includer. It reaches high maximum Na^+ and Cl^- levels in the leaves rapidly, i.e. already at a concentration of 200 mM NaCl in the root medium without further increase as concentrations of applied NaCl are step-wise increased up to 400 mM during subsequent days. Under a similar regime of NaCl supply roots of young seedlings of *Cereus validus* gradually accumulated Na^+ and Cl^- but the levels reached at 400 mM NaCl after 9 days were only a sixth to a fourth of those attained in leaves of *M. crystallinum*. Roots of *C. validus* largely excluded NaCl from the xylem stream because stem levels remained rather low throughout the entire experiment and were 40 to 60 times lower than in the leaves of *M. crystallinum*. A comparison of different cacti is given in Figure 3. *Opuntia humifusa* seems to be a still more effective salt excluder than *C. validus* and its inland population was more effective than the coastal one.

16.3.2 MORPHOLOGICAL ROOT-SYSTEM DYNAMICS *VERSUS* ROOT-SYSTEM SIGNALING

We have noted above that cacti may accumulate NaCl at the root level but exclude it there from transport to the shoot. During longer exposure the absorptive fine roots of the *C. validus* seedlings described (Figures 2 and 3) died and even the basis of the

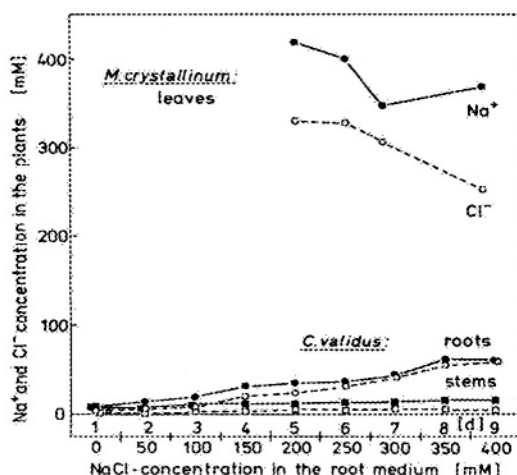


Figure 2. Concentrations of Na^+ (closed symbols) and Cl^- (open symbols) in leaves of *Mesembryanthemum crystallinum* and in roots (circles) and stems (squares) of seedlings of *Cereus validus* where each day (upper scale of the abscissa) NaCl concentration of the root medium (lower scale of the abscissa) was increased by 50 mM. Drawn from the more extensive data sets of Heun *et al.* (1981; *M. crystallinum*) and Nobel *et al.* (1984; *C. validus*) to the extent the different experiments allowed comparison. In *M. crystallinum* Na^+ and Cl^- concentrations in the leaves remained high at a constant level for more than 3 weeks, the fluctuations seen here are not significant (Heun *et al.*, 1981).

stems became necrotic. In many of these cases the NaCl solution was diffusing upwards in the dead tissue gradually killing the whole seedlings from bottom to top after several months. However, in other cases the stem basis entirely dried out rapidly, and then the remaining healthy part of the stem became totally insulated from the saline substratum. Their constitutive CAM kept them alive under this condition with recycling of respiratory CO_2 by fixation via PEPC into malate during the dark periods and re-assimilation of CO_2 via RUBISCO during the light periods with stomata continuously closed (Lüttge, 1993, 1997). When water is available again, cacti can regrow adventitious roots quite rapidly (Kausch, 1965), and the dynamics of drying and rewetting cycles in desert soils also differ for maintenance of meristems and lateral root growth in deeper and more peripheral parts of the root system (Dubrovsky *et al.*, 1998). Thus, in seasonal cycles of salinity and/or drought stress and rainfall cacti can close up and use CAM with solar energy input overcoming the besiegement by soil salinity.

Function of the root system of the annual *M. crystallinum* in response to salinity is totally different from the versatile advance / retreat strategy of absorptive fine roots observed in the cacti. In addition to transport of large amounts of NaCl via the roots to the shoots the roots must also have signaling functions. We have noted already above that in *M. crystallinum* salinity induces a shift from C_3 -photosynthesis to CAM. Winter and Gademann (1991) have suggested that repeated day-time reductions of turgor due to transpiratory loss of water when the plants are still in the C_3 -state may trigger a signal-transduction mechanism which eventually causes the shift in mode of photosynthesis several days after the onset of salinity stress. Conversely it was argued

that, while this may possibly play a certain role, the major signal for the shift comes from the roots (Michalowski and Bohnert, 1992; Eastmond and Ross, 1997). Indeed,

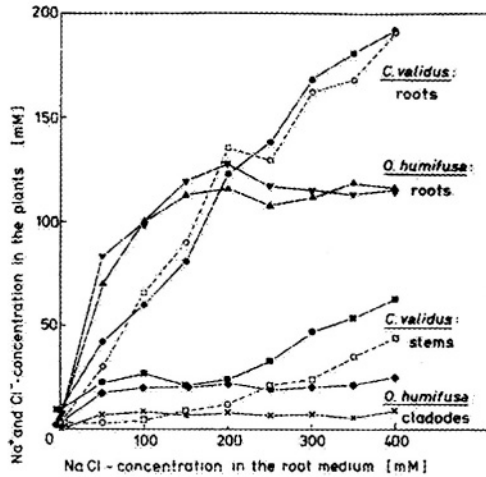


Figure 3. Concentrations of Na^+ (●, ■) and Cl^- (○, □) in roots and stems of *Cereus validus* seedlings and concentrations of Na^+ in roots and cladodes of rooted *Opuntia humifusa* cladodes from a coastal (▼, ◆) and an inland (▲, ×) population. The seedlings of *C. validus* were stressed for 2 weeks and the rooted cladodes of *O. humifusa* for 6 weeks with NaCl-concentrations in the root medium as indicated on the abscissa. Curves were drawn from data in Nobel *et al.* (1984; *C. validus*) and Silverman *et al.* (1988; *O. humifusa*). For *O. humifusa* concentrations of Na^+ in the root and cladode tissue, respectively, were calculated from the dry weight based data assuming average water contents of roots and stems/cladodes of cacti of 75% and 90%, respectively (P.S. Nobel personal communication).

three growth regulators, namely abscisic acid (ABA), farnesol (an ABA analog) and the cytokinin benzylaminopurine, were found to substitute for NaCl in induction of CAM when fed to plants in nutrient media (Dai *et al.*, 1994). Application of exogenous ABA at concentrations of 5 – 10 μM led to induction of CAM and application was more effective when ABA was added to the root medium rather than given to the leaves. The effects depended on plant age and ABA concentrations; higher concentrations tended to be inhibitory (Edwards *et al.*, 1996; Chu *et al.*, 1990). Most conclusions still come from experiments with exogenous phytohormone applications, and much still needs to be done to clarify the signal transduction chain from soil salinity to expression of CAM in the leaves. Thomas *et al.* (1992 b) note that while salt induced increases of PEPC levels in the leaves, i.e. the key enzyme of CAM, are kinetically correlated with ABA and cytokinin levels they are not related to them as primary causes. Nevertheless, the primary involvement of roots in a signaling system is highly likely as it is now so well established for many drought effects in intact whole plants (Davies and Zhang 1991; Zhu and Zhang, 1997).

16.3.3 CONSTITUTIVE CAM *VERSUS* INDUCIBLE CAM

Induction of CAM as it occurs in *M. crystallinum* immediately poses the question of reversibility. In *M. crystallinum* induction of CAM by salt, drought and osmotic stress in the root medium is only partially reversible which also depends on the age of the plants because in addition to the stress response there is a developmental program in *M. crystallinum* which leads to expression of CAM as the plants age (Winter, 1973, 1974 a, b; Winter and Lüttge, 1979; Lüttge, 1993; Ratajczak *et al.*, 1994). Quite obviously this is appropriate for the life cycle of the annual plant. It germinates in its natural habitat after sufficient rainfall, uses the more productive C₃-mode of photosynthesis for built-up of its biomass in the early stages of growth and then switches to CAM as the dry period and soil salinity develops. This is always naturally correlated to its aging and allows it to bring a large amount of seeds to maturity with some contrast to co-occurring C₃-annuals that die earlier (Winter *et al.*, 1976, 1978). Having thus completed its life cycle and starting anew with seed germination when the next wet season relieves drought, there is no adaptive advantage of a strict reversibility of CAM induction in this annual.

This is very different for perennial species, which use their photosynthetic apparatus once it is established in repeated cycles of dry and wet seasons. They must either make their choice, which then will prove of adaptive advantage only for one set of conditions, or, indeed, they must be highly versatile with reversible shifts between the modes of photosynthesis.

The latter is the case in the leaves of many species of the tropical shrub and tree genus *Clusia*. They may switch modes of photosynthesis within hours (Lüttge, 1996). Although *Clusias* can occur at coastlines on rocks as well as in sand dunes, i.e. in the restinga of Brazil (Franco *et al.*, 1996; de Mattos *et al.*, 1997), and are certainly subject to some salinity at least via salt spray (Reinert *et al.*, 1997), this may not be much related to salt stress. Soil salinity is low in the restingas (Reinert *et al.*, 1997). In their various tropical habitats *Clusias* are subject to very strong changes in insolation and water availability which are not only seasonal but often quite rapid (Lüttge 1995, 1996). Thus, if C₃-photosynthesis and CAM indeed are more favorable under one and the other set of conditions, respectively, quick switches may constitute an adaptive advantage.

It is worth mentioning this in contrast to the C₃-CAM-switch in *M. crystallinum* with its only limited and partial reversibility and the constitutive CAM in the cacti. For the more stable dry environment of the major distribution of the latter constitutive CAM appears to be a good adaptation.

The contrast between the slow and only partially reversible switch in *M. crystallinum* and the rapid shifts in *Clusias* poses very interesting questions. At a first glance the latter appear to be more readily understandable. It has often been noted that there is nothing much special about the enzymology of CAM. Even key enzymes of CAM, such as PEPC, acid decarboxylating enzymes, the tonoplast ATPase energizing vacuolar malic-acid accumulation and others are also well known house-keeping enzymes in C₃-plants serving basic cell functions. (Cockburn, 1983; Cushman and

Bohnert, 1996; Lüttge, 1998). Hence, CAM induction may rather be a rearrangement of the major flows of carbon than an establishment of new enzymatical complements. Such rapid rearrangement may in fact occur during fast switches. On the other hand, the switch in *M. crystallinum*, which now rapidly is increasingly well understood at the molecular levels of genes, polynucleotides and enzyme proteins (Chapters 17 and 22), is clearly based on regulation of gene expression leading to increased levels of CAM key-enzymes and modified isoenzyme complements. Uptake and accumulation of NaCl precedes CAM-expression by several days. Nevertheless, in these studies it is often quite difficult to find out if the molecular changes are associated with salinity *per se* and the considerable accumulation of NaCl (Figure 2) or with the requirements of CAM. Although *M. crystallinum* in its own right now evolves to become the model plant of stress physiology as the endeavor advances to sequence its entire genome (Walbot, 1999), which having only 390,000 kbp is only about 3 times larger than that of *Arabidopsis thaliana* (Bohnert *et al.*, 1988), it appears to be highly worthwhile to study the at least partially different mechanisms of C₃/CAM switches in the perennial species for comparison at the molecular level as well.

For comparison with *M. crystallinum* the cereal *Sorghum bicolor* offers another interesting case (see Chapter 14). It appears that salt stress can amplify the potential for C₄-photosynthesis in some C₄ grasses (e.g. activities of PEPC) such as *Sorghum* (Amzallag *et al.*, 1990 b) and *Aeluropus littoralis* (Shomer-Ilan and Waisel, 1973). In *Sorghum* it is the tolerance of salinity itself, however, which can be induced in some cultivars, and this is possible only in a rather narrow genetically defined developmental window. A pretreatment at moderate levels of salinity, i.e. above a threshold of 30 mM NaCl and up to 150 mM, induces adaptation to more strong salt stress (300 mM) applied subsequently. This, however, only works effectively when the pretreatment is applied 5 days after germination, the adaptation decreases when the pretreatment occurs later. The effect of the pretreatment is modulated by addition of phytohormones, it is accelerated by ABA and inhibited by cytokinin and gibberellic acid (Amzallag *et al.*, 1990 b, 1992, 1993).

16.3.4 CENTRAL VERSUS PERIPHERAL WATER STORAGE TISSUE

Cacti have central water storage parenchymas in their stems. The epidermal bladders of *M. crystallinum* densely covering leaves, stems and seed receptacles have been considered by Haberlandt (1904) to be a peripheral water storage tissue. Although bladders of *M. crystallinum* occasionally may contain plastids (Lüttge *et al.*, 1978) in both cases the water storage tissues are essentially non-photosynthetic and do not participate in the day/night-oscillations of malic-acid levels during CAM (Lüttge *et al.*, 1989 a; Winter and Lüttge, 1976), their main function really being provision of water reservoirs.

For the central water parenchyma of cacti it is perhaps more obvious than for the peripheral water storage tissue of *M. crystallinum* that it may provide water to the metabolically active tissue under stress. Nevertheless, it must be clear in addition that in cacti even during situations of besiegement as described above, when stomata are closed day and night and metabolism is idling with solar energy input driving internal

carbon recirculation, some water must be lost slowly via cuticular transpiration (Lüttge *et al.*, 1989 a). It was shown that cacti can loose up to a little more than 50% of their whole tissue water until this becomes lethal (Holthe and Szarek, 1985). Larger cacti have a better surface to volume ratio and a relatively larger water storage volume giving a larger capacity to buffer losses. Clearly the major problem then is seedling establishment, and one would expect to find age classes of water-storing CAM plants in the field depending on the variable expression of wet and dry seasons, which every now and then allows more seedling survival (Jordan and Nobel, 1979, 1982).

The possible functioning of the bladders in *M. crystallinum* initially was more perplexing. As noted above the search for salt concentrating bladders laid the ground for the serendipitous discovery of CAM-induction by salinity in *M. crystallinum*. It is often misunderstood and misquoted in the literature but quite clearly its bladders do not function for salt accumulation concentrating NaCl (Figure 1). After this was discovered biophysical studies using the cellular pressure probe addressed the question if in fact they can serve as external water reservoirs for the metabolically active mesophyll as suggested by Haberlandt (1904). This function, indeed, is supported by the comparatively high hydraulic conductivity (L_p) of the membrane between the bladder cells and the underlying photosynthetically active cells and the volume and pressure dependence of their cell wall volumetric elastic modulus (ϵ), which is positively correlated with volume and pressure (Steudle *et al.*, 1975, 1977). The bladders tend to become larger and to cover the leaves and stems more densely as the plants are subject to salinity (Figure 4), the whole plants ramify more strongly and leaves are smaller and more succulent. Turgor pressure is somewhat reduced as the bladders are filled with saline solutions under salt stress (Figure 5).

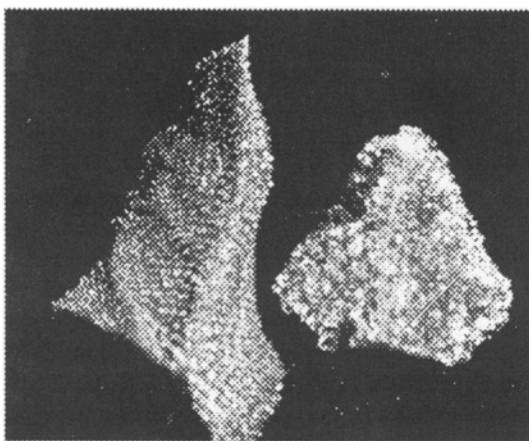


Figure 4. Leaves of *Mesembryanthemum crystallinum* grown without a NaCl load (left) and under NaCl salinity (right). (See Lüttge *et al.*, 1978).

The daily dynamics of water movements between bladders and mesophyll of *M. crystallinum* during CAM have been studied by Rygol *et al.* (1986, 1987, 1989;

Figure 6). As a result of nocturnal malic-acid accumulation in the mesophyll cells osmotic pressure (π) was highest at the end of the dark period and declined during the day as malic acid was consumed again. Although they do not participate in the malic-acid rhythm of CAM (see above), the bladders followed this trend of π in the morning, but π already began to increase again by the end of the day. This must be due to water movements. The osmotic part of the driving force for water ($\Delta\pi$) in the morning and at midday was directed from the bladders of the upper, adaxial epidermis towards the

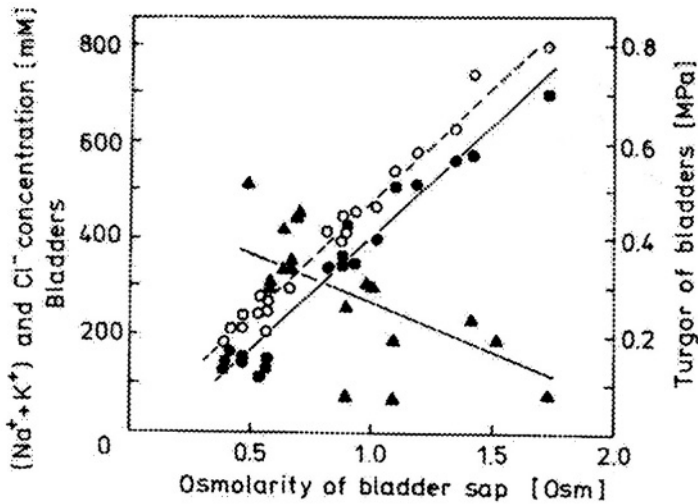


Figure 5. Relations of $\text{Na}^+ + \text{K}^+$ concentrations (○), Cl^- concentrations (●), turgor pressure (▲) (P) and osmolarity (π) in the sap of bladders of *M. crystallinum* grown at different NaCl-concentrations in the root medium (after Lüttge *et al.*, 1978; Winter and Lüttge, 1979).

mesophyll. The same was found for the bladders of the lower, abaxial epidermis at midday, but for these bladders there was a small gradient in the opposite direction at dusk. At the end of the day π was rather similar in all the three tissues, with a small osmotic driving force from the mesophyll towards the bladders on both leaf surfaces. Turgor pressure (P) in the mesophyll cells was also highest at the end of the night due to osmotic water uptake driven by the accumulated malic acid, and it also declined during the day. The day-night changes of P in the bladders, however, were shifted in phase compared with the mesophyll; turgor increased until midday and then decreased again towards the evening. This was much more pronounced in the bladders of the upper epidermis than in those of the lower epidermis. The hydrostatic part of the driving force (ΔP) for water-movement at noon and dusk was directed from the bladders to the mesophyll, and at noon this added up to the osmotic driving force, while at dawn P was rather similar in all three tissues. The water potential (ψ), i.e. the total driving force for water movement ($\Delta\psi$), is given by $\psi = P - \pi$. It largely is determined by π alone, since $\pi > P$ (see Figure 6). There was a strong overall driving

force from both bladder layers towards the mesophyll at noon and also from the bladders of the upper epidermis at dawn. The phase shift between diurnal changes of the water relations parameters in the mesophyll tissue and the bladder-cell layers offers a precise indication of the water movements between these tissues. In conjunction with the osmotically important oscillations of malate levels in the mesophyll during CAM the water reserves in the bladders can support the water-relations of the mesophyll.

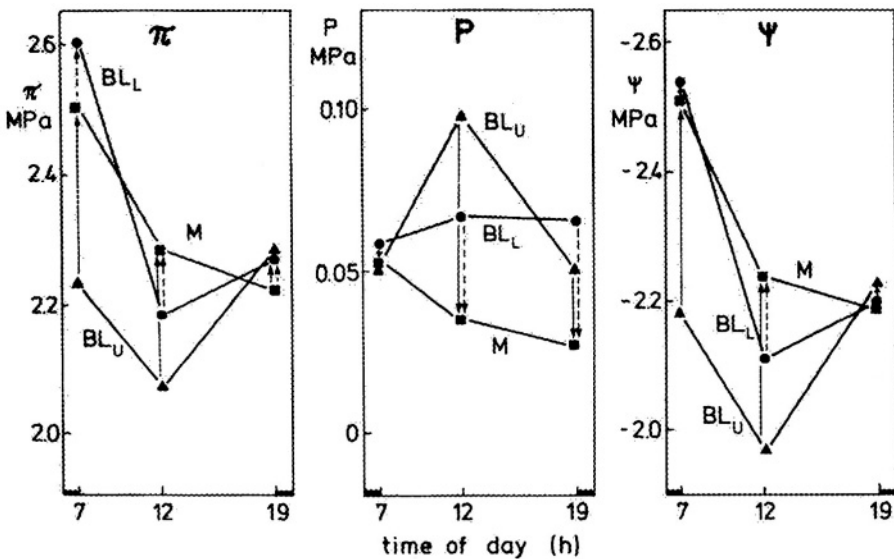


Figure 6. Daily course of osmotic pressure (π), turgor pressure (P) and water potential ($\psi = P - \pi$) in bladders of the upper (adaxial, BL_U) and the lower (abaxial, BL_L) epidermis and the mesophyll cells (M) of leaves of *M. crystallinum*. Arrows indicate the direction of the respective driving forces, i.e. $\Delta\pi$, ΔP and $\Delta\psi$, for water movement. Drawn after data of Rygol *et al.*, 1989.

16.4 Organization at the whole plant level, developmental programs, phylogeny: do they relate to the poor diversity of plants with the photosynthetic C_4 -carboxylation mode in saline habitats?

What do we learn from all of this for understanding the apparent paradox that there is so low diversity of plants with the C_4 -carboxylation mode of photosynthesis in saline habitats? Perhaps the expectation is wrong and based on unjustified mixing up of drought stress, salt stress and osmotic stress?

Most genuine halophytes are salt includers and they are succulent, they show „salt succulence“. Salt is compartmented in large central vacuoles and diluted by osmotic uptake of water. C_4 -plants require a special anatomy with green mesophyll and bundle-sheath tissues for the division of labor between primary CO_2 -fixation via PEPC and final CO_2 -assimilation via RUBISCO, respectively. Does this prevent the development of leaf succulence? In fact, where C_4 -plants are halophytic they have a different mode of coping with the salt, e.g. the elimination of salt by excretion into large vacuoles of

salt bladders in the genus *Atriplex* (Figure 1) or to the outer leaf surface via salt glands as in some C_4 grasses (Pollak and Waisel, 1970).

Conversely, the very trait of succulence in CAM then should be really favorable for growth under salinity stress. However, the case of cacti in salinas as described above is not pertinent. It is a simple case, since these cacti are salt excluders and thus stress avoiders. They do not tolerate salinity stress but tolerate the periodically saline habitat with morphological root-system dynamics and CAM as a method to overcome limited periods of blockades by stress.

For salinity tolerance by salt inclusion in higher plants not much sophistication seems to be required at the morphological and anatomical levels of organization. In fact, cell suspension cultures have been very successful for establishing salt tolerant lines and for studying salinity responses *in vitro* (Lüttge, 1983). This also applies to *M. crystallinum* (Chapter 17). All the traits making it halotolerant appear to be expressed at the simple cell level of organization. This includes for instance synthesis of the compatible solutes (Chapter 9) proline and pinitol, which are induced in *M. crystallinum* by salinity (Demmig and Winter, 1986; Treichel *et al.*, 1984; Thomas *et al.*, 1992 a; Yen *et al.*, 1995; Vera-Estrella *et al.*, 1999). However, CAM is not induced (Thomas *et al.*, 1992a; Yen *et al.*, 1995) or remains very weak at the most (Kluge *et al.*, 1987; Treichel *et al.*, 1988) in NaCl-challenged cell suspension and callus cultures of *M. crystallinum*. Several polypeptides which are induced by salinity in light-grown green callus must all be related to salt tolerance and not to CAM (Yen *et al.*, 1997).

Although excised leaves of constitutive CAM plants are known to continue to show CAM oscillations for some while no CAM performing cell cultures are available to date despite many attempts of their establishment. This is somewhat astonishing because the anatomy of CAM leaves, e.g. in the genus *Kalanchoë*, is often very simple with only one type of large spherical and highly vacuolated cells. Moreover, CAM continues in the absence of stomatal control, when the epidermis of *Kalanchoë* is peeled (Kluge and Fischer, 1967), so that this part of leaf differentiation is not required. Nevertheless, CAM quite generally seems to require the whole-plant level of organization. In the case of *M. crystallinum* there is an ongoing debate of whether CAM induction is only a matter of stress or a developmental program is also essential (e.g. Piepenbrock and Schmitt, 1991; Schmitt and Piepenbrock, 1992 *versus* Cushman *et al.*, 1990; Herppich *et al.*, 1992). This is a basically important question in view of our discussion of the organizational level required for the expression of CAM. It appears that both in fact are involved and that the developmental program is amplified by stress (Ratajczak *et al.*, 1994). The stress required is not specific. It is not the straightforward ionic stress of NaCl, as it may in fact be the dominant signal for many halophytes and for the expression of salt-tolerance genes in cell suspension cultures (Yen *et al.*, 1997). Other salts, hypoxia in the root medium, osmotic stress and drought also induce CAM in intact plants of *M. crystallinum* (Winter, 1973, 1974 a, b; Winter and Lüttge, 1979).

Salt-induced changes from C_3 -photosynthesis to CAM in *M. crystallinum* are dependent on leaf age (Winter, 1973, 1974 a; Winter and Lüttge, 1979). Even an age dependent shift to CAM is observed in the absence of salinity stress (Ratajczak *et al.*,

1994). As mentioned above phytohormones (ABA, cytokinins) may be involved as well as photoperiod and the phytochrome system acting synergistically with salt stress and accelerating developmental programs (Cheng and Edwards, 1991; Cockburn *et al.*, 1996). The reversibility of NaCl-induced CAM is age-dependent (Ratajczak *et al.*, 1994); CAM induction is stronger and reversibility is lower as leaves age (Winter, 1974 a).

Although in constitutive CAM plants CAM can proceed when leaf epidermis with stomata is peeled (see above; Kluge and Fischer, 1967) and CAM also occurs in green aerial roots of epiphytic orchids lacking stomata (Winter *et al.*, 1985), some recent studies of stomatal regulation in *M. crystallinum* may still hide very important hints with respect to organization and CAM expression (Tallman *et al.*, 1997). Stomatal regulation is generally a very complex process governed by a sophisticated signal transduction network. For CAM it is often assumed that day-time stomatal closure is supported by the high internal CO₂-concentrations during organic acid remobilization and decarboxylation (Cockburn *et al.*, 1979). Of course, the situation also in CAM must be more complex than that allowing regulation of stomatal opening in the other CAM-phases (dark period, transitions in the morning and afternoon; Osmond, 1978). In *M. crystallinum* after the C₃-CAM transition guard cells loose the opening response to blue and white light which is expressed in guard cells in the C₃-state. They also loose light-dependent zeaxanthin formation. Zeaxanthin appears to be involved in the signal transduction chain between light and stomatal response (Tallman *et al.*, 1997).

Is *M. crystallinum* a very unique case then? It does not really present itself as a straightforward facultative halophyte where halophytism is linked to the expression of CAM. It rather presents itself as responsive to general stress situations with a developmental program that is synchronized with the climatic conditions prevailing in its natural environment (Winter *et al.*, 1976, 1978). The genome analysis of *M. crystallinum*, which is under way (Chapter 17), and comparative studies of other C₃/CAM intermediate species will soon tell us more about this.

Are we left then with the enigma that C₄-photosynthesis to some extent and CAM very clearly only play a very limited role in saline habitats? We may! However, phylogeny may give us a clue, at least with respect to CAM. The origin of CAM certainly is polyphyletic (Moore, 1982; Lüttge, 1987; Monson, 1989; Raven and Spicer, 1996, Winter and Smith, 1996 b). Possibly CAM has evolved primarily as a CO₂-concentrating mechanism (Griffiths, 1989; Ehleringer and Monson, 1993; Winter and Smith, 1996 b). It also occurs in aquatic plants in fresh water, where it has nothing to do with salt, osmotic or drought stress (Keeley, 1996; Raven and Spicer, 1996). Then, as a consequence of concentrating CO₂ water saving and water acquisition secondarily may have proven to be of adaptive advantage. Is CAM thus too young phylogenetically, to have also made it already for salinity? Is *M. crystallinum* ahead due to the rapid speciation in the Aizoaceae-family (Winter and Smith, 1996 b)? Edifices of logical phylogenetical speculations are often built up readily. However, with gene analyses and molecular timing phylogeny now strongly develops towards a hard experimental science and this will tell us more in the future.

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CHAPTER 17

INDUCTION OF CRASSULACEAN ACID METABOLISM BY SALINITY – MOLECULAR ASPECTS

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Abstract

The serendipitous discovery that salinity stress can induce a switch from C_3 photosynthesis to Crassulacean acid metabolism (CAM) in the Aizoaceae *Mesembryanthemum crystallinum* has instigated a wide range of ecophysiological, biochemical, and molecular genetic studies aimed at understanding the mechanistic basis of this phenomenon. The underlying changes in gene expression that initiate CAM induction are influenced by the interaction of environmental and developmental factors resulting in an extremely plastic adaptation. *M. crystallinum* holds the notable distinction of being one of the few true halophytes among inducible CAM species. The peculiarity has resulted in *M. crystallinum* becoming a favored model for genetic and molecular genetic studies.

17.1 Introduction

The fortuitous detection nearly three decades ago of a salinity-induced shift in photosynthesis from C_3 to CAM in *Mesembryanthemum crystallinum* L. (Aizoaceae, Caryophyllales), the common ice plant, (Winter and von Willert, 1972; see Chapter 16) has had far-reaching consequences for plant biology. Previous ice plant research has provided a bountiful harvest of knowledge, and present prospects are even more exciting. Physiological effects of salinity have been studied in various CAM species (Chapter 16), but studies on the molecular basis of CAM induction by salt stress have focused almost exclusively on *M. crystallinum*. In addition, most of the early work on salinity stress responses was initiated with the ice plant. In recent years, researchers have emphasized various aspects of CAM including ecological physiology, evolution, biochemistry, and regulation by environmental factors (Cockburn, 1985; Ting, 1985; Winter, 1985; Lüttge, 1987; Griffiths, 1988; Monson, 1989; Leegood and Osmond, 1990; Griffiths, 1992; Smith and Bryce, 1992; Ehleringer and Monson, 1993; Winter and Smith, 1996; Cheffings *et al.*, 1997). Work has begun on molecular genetic aspects

of CAM – including the regulation of enzyme activity, protein synthesis profiles, and transcriptional activation or repression leading to altered gene expression patterns (Lüttge, 1993; Cushman and Bohnert, 1996, 1997, 1999). Studies of the ice plant have, however, also enhanced our knowledge of carbohydrate synthesis, storage and transport, photosynthesis protection, drought and salinity stress responses and ecological distribution (Winter and Smith, 1996; Adams *et al.*, 1998; Cushman and Bohnert, 1999).

More recently, the extreme plasticity with which *M. crystallinum* developmentally responds to environmental stresses has stimulated interest in understanding the signal transduction processes leading to these responses. We will emphasize this emerging topic in our discussion, and we will discuss the uses of *M. crystallinum* as a genetic model as a result of our work. Current work in our laboratories with *M. crystallinum* centers on large-scale gene characterization and discovery of novel genes, the further development and exploitation of the plant as a genetic model system, and the development and application of transformation and regeneration procedures (Cushman *et al.*, 1999 a; Cushman and Bohnert, 1999). Such advances will be essential in order to dissect the molecular basis of CAM and salinity tolerance in this remarkable halophyte.

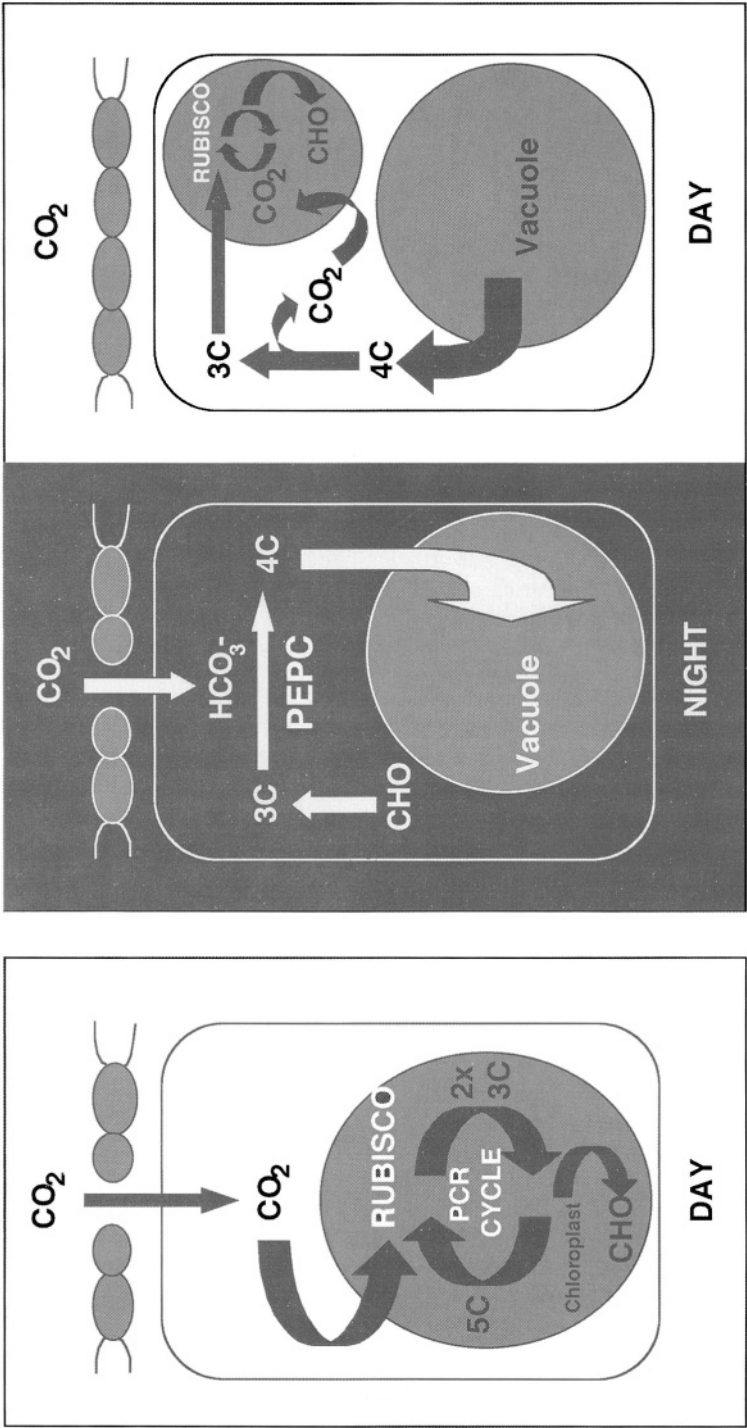
17.2 Crassulacean Acid Metabolism

Most plants assimilate atmospheric CO_2 through the C_3 photosynthetic pathway using ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), but approximately 10% of higher plant species use alternative strategies for sequentially assimilating and concentrating atmospheric CO_2 . These alternatives are C_4 photosynthesis and Crassulacean acid metabolism (CAM). Both alternatives utilize as a primary “ CO_2 pump” the enzyme phosphoenolpyruvate carboxylase (PEPC) that ultimately elevates intracellular CO_2 concentrations in the vicinity of Rubisco. C_4 plants spatially separate the two carboxylation reactions between distinct cell types, mesophyll and bundle sheath cells with distinct gene expression patterns (Furbank and Taylor, 1995; Ku *et al.*, 1996). In contrast, CAM plants perform both reactions within the same cell type, but separate primary and secondary CO_2 fixation between night and day, respectively (Figure 1). By conducting the bulk of atmospheric CO_2 uptake and assimilation at night when evapotranspiration rates are low and limiting stomatal opening during the day, water loss is minimal and water use efficiency is improved. Both C_4 photosynthesis and CAM operate to overcome limitations in CO_2 supply arising from environmental factors such as high light and high temperature, or low water availability, which can lead to

Figure 1. Metabolite Flux During CAM. Carbon flow in a cell performing C_3 photosynthesis (Left panel) and in a cell performing CAM during the day and night (right panel). In C_3 photosynthesis, atmospheric CO_2 is fixed by Rubisco via the photosynthetic carbon reduction (PCR) cycle within the chloroplast. In CAM, nocturnal CO_2 uptake through open stomata and glycolytic breakdown of storage carbohydrates (CHO) from chloroplastic or extrachloroplastic sources results in the formation of PEP which serves as the substrate for CO_2 fixation via bicarbonate (HCO_3^-) by phosphoenolpyruvate carboxylase (PEPC) leading to C_4 acid (mainly malate) production and storage within the vacuole. During the day, stomata close and fixation of external CO_2 is replaced by malate decarboxylation and refixation of released CO_2 by Rubisco and the PCR cycle. Pyruvate and PEP (3C) fuel gluconeogenesis to regenerate storage carbohydrate pools within the chloroplast or other extrachloroplastic stores. The contribution of mitochondria to malate formation and decarboxylation is not shown for simplicity (see color version in the color section).

CRASSULACEAN
ACID METABOLISM

C3 PHOTOSYNTHESIS



excess photorespiration based on the incomplete discrimination of CO_2 over O_2 by Rubisco.

The driving forces responsible for CAM evolution most likely include limited CO_2 availability in past geological eras due to periodic decreases in global atmospheric CO_2 concentrations, high light and temperature conditions, lack of water, or a combination of these factors (Ehleringer and Monson, 1993; Raven and Spicer, 1996). However, the existence of CAM plants in low- CO_2 aquatic habitats suggests that CAM arose primarily as a CO_2 concentrating mechanism and that limited CO_2 may have provided the primary selective pressure for the evolution of CAM (Osmond, 1978; Cockburn, 1985; Ehleringer and Monson, 1993; Raven and Spicer, 1996; Keeley, 1996, 1998).

Improved water use efficiency may have provided an additional, but secondary, advantage that allowed colonization of diverse ecological niches with unreliable water availability or long-term water deficits (see also Chapter 15). The genetic modifications required to perform CAM are considered less extensive than those needed for the differentiated cell types in C_4 plants (Ku *et al.*, 1996). In support of such a view are recent phylogenetic studies suggesting that CAM evolved earlier than C_4 photosynthesis (Gehrig *et al.*, 1998). Nevertheless, the emergence of CAM required many genetic modifications in diurnal or circadian regulatory systems, in intracellular partitioning of photosynthetic products, adjustments in environmental responses, and changes in metabolic signaling to temporally regulate two competing carboxylation reactions within a single cell type.

17.3 *Mesembryanthemum crystallinum* – a model for stress adaptation

Research on *M. crystallinum* has contributed greatly to our understanding of the physiology, biochemistry, and molecular genetics of CAM and has become synonymous with many other stress responses studied at the molecular level (Bohnert *et al.*, 1988, 1994, Cushman and Bohnert, 1996, 1997, 1999). The plant is native to southern and eastern Africa (Winter *et al.*, 1978; Winter and Troughton, 1978; Bloom and Troughton, 1979). It has been introduced into western Australia, Mediterranean coastal regions, the Caribbean, and along the coasts of the western United States, Mexico, and Chile (Winter and Smith, 1996). In its native habitat, the plant germinates and becomes established after a short (winter) rainy season, followed by progressive drought stress coupled with increasing salinity (Winter *et al.*, 1978; Bloom and Troughton, 1979). Evolutionary adaptations that can be defined in terms of anatomical, physiological, biochemical and molecular processes assure reproductive success under such hostile conditions. The seedlings must initially develop a critical mass of plant material, with the formation of up to seven pairs of primary leaves. The onset of stress at the end of the rainy season then accelerates the developmental shift from a juvenile growth to a mature, reproductive growth habit characterized by smaller, succulent leaves along developing side shoots that redifferentiate into floral meristems. The production of side-shoots and the smaller leaves in mature plants coincides with the appearance of CAM in the entire plant. *M. crystallinum* has a survival advantage in its ability to conduct both C_3 and CAM and its ability to modulate the use of each pathway depending on prevailing water availability conditions. The plants, under optimal, well-watered conditions, may grow

for a long time in C_3 mode and gradually shift into CAM, but the timing and magnitude of flowering and seed set depends on the performance of CAM and on the size the plant had achieved during unstressed growth (Winter and Ziegler, 1992). Following flowering, older portions of the plant progressively die off, while the developing seed capsules remain turgescient until the seeds have matured.

M. crystallinum's traits make it one of the most attractive models for investigating the molecular basis of stress adaptations. It is a rapidly growing, self-fertile annual with a relatively small genome (390 Mb in nine chromosomes; $2N = 18$), which is only about three times larger than that of *Arabidopsis* (DeRocher *et al.*, 1990; Adams *et al.*, 1998) and produces large quantities of small seeds. Furthermore, it is possible to accelerate the normal life cycle of *M. crystallinum* from five months in its natural habitat (Winter *et al.*, 1978) to only 7 weeks under long photoperiod conditions (Cheng and Edwards, 1991). Extended photoperiods miniaturize the plants - an important consideration when conducting genetic screening studies. Mutant collections have been established in *M. crystallinum* from fast neutron and gamma-ray irradiated or chemically treated (EMS, ethylmethane sulfonate) plants (Adams *et al.*, 1998). Selection schemes for the isolation of plants defective in CAM or sensitive to salinity stress have been developed (Cushman *et al.*, 1999 a). Transgenic hairy-root cultures, callus tissue, and cell suspensions from *M. crystallinum* (Andolfatto *et al.*, 1994; Ishimaru, 1999; J.C. Thomas, T. Wulan, and J.C. Cushman, unpublished) have been recovered following transformation by *Agrobacterium rhizogenes* and *A. tumefaciens*, respectively. Regeneration systems for *M. crystallinum* provide the experimental platform for future transgenic analysis aimed at dissecting the functioning of cellular and organismal stress tolerance mechanisms (Meiners *et al.*, 1991; Abou-Mandour, 1992; Wang and Lüttge, 1994; Cushman *et al.*, 2000).

M. crystallinum has provided indispensable insights into the physiological and biochemical mechanisms of salinity tolerance mechanisms. Salinity and drought affect a suite of sugar alcohol compatible solutes (i.e., solutes that will not interfere with biochemical reactions even at high concentration; Chapter 9) including inositol, ononitol, and pinitol (Paul and Cockburn, 1989; Vernon and Bohnert, 1992 a; Ishitani *et al.*, 1996; Nelson *et al.*, 1998 a, b; 1999). The activities of ion transporters and specific water channel proteins in roots, stem and leaves are adjusted under stress conditions (Kirch *et al.*, 2000; Su *et al.*, 2001). At the molecular level, some genes are controlled transcriptionally by salt stress at all periods of development. Two of these are *Imt1*, encoding a *myo*-inositol O-methyltransferase, the key enzyme leading to the production of compatible solutes (Vernon and Bohnert, 1992; Ishitani *et al.*, 1996), and *Atpc* encoding the V-ATPase c-subunit responsible for energizing solute partitioning into the vacuole (Tsiantis *et al.*, 1996). In contrast, the CAM isoform of PEPC (encoded by gene *Ppc1*) and other CAM-related genes are not inducible in young juvenile plants (Bohnert *et al.*, 1994). These genes become progressively transcriptionally inducible (Ostrem *et al.*, 1990; Cushman *et al.*, 1990) as the juvenile leaves mature and the genes are constitutively expressed in mature leaves (Cushman and Bohnert, 1996).

Halophytic cell suspension cultures have been recovered from *M. crystallinum* leaf tissues which mimic intact plant Na^+ , pinitol, and IMT1 protein accumulation patterns as well as increased activities of vacuolar (V-ATPase) and plasma membrane (P-ATPase) H^+ -ATPases (Vera-Estrella *et al.*, 1999). Such cell suspension cultures, like

juvenile plants, have a limited capacity for sodium transport into vacuoles (Löw *et al.*, 1996; Barkla *et al.*, 1995; Tsiantis *et al.*, 1996; Vera-Estrella *et al.*, 1999). In adult plants, specialized epidermal bladder cells (EBC, which serve as water storage reservoirs, accumulate sodium ions at concentrations similar to photosynthetic cells (Lüttge *et al.*, 1978; Lüttge, 1993). Other reports have indicated that sodium concentrations in the EBC exceed those in the remainder of the leaf or shoot (Adams *et al.*, 1992; Adams *et al.*, 1998). The enhanced accumulation of Na^+ and pinitol in EBCs correlates well with tonoplast Na^+/H^+ antiport and V-ATPase H^+ -transport activities which are highest in these cells (Barkla *et al.*, 2000). There is, even for large adult plants, a limit to the capacity for sodium uptake, and this eventually arrests further development.

The characterization and expression of several gene families have been related to developmental stage and magnitude of environmental stress. Among those are the families encoding the small subunit of RUBISCO (*RbcS*; DeRocher and Bohnert, 1993), water channel proteins (*Mips*; Yamada *et al.*, 1995; Kirch *et al.*, 1999), CAM metabolism genes (Cushman and Bohnert, 1996), transcripts for polyol biosynthesis (Ishitani *et al.*, 1996, Nelson *et al.*, 1998, 1999) and photorespiration (F. Quigley and C.B. Michalowski, unpublished data). We are only beginning to realize the practical benefits of the characterization of genes that seem to be at the basis of *M. crystallinum's* capability for tolerating highly saline solutions during some time of its life cycle. Gene transfer and the alteration of expression patterns of genes encoding biosynthetic enzymes for compatible solutes confer slightly enhanced salinity tolerance in tobacco (Sheveleva *et al.*, 1997, 1998, 1999). We have now begun an organized search for additional genes - in principle a search for all genes with a relationship or correlation to salinity stress tolerance - to determine their suitability for transfer into other plants (Bohnert and Jensen, 1996 a, b; Bohnert and Sheveleva, 1998; Nelson *et al.*, 1998).

17.4 CAM induction

17.4.1 PERMUTATIONS AND PLASTICITY

The induction of CAM is a complex process that integrates external environment and development under the control of hormonal effects. Many criteria have been used to define CAM induction such as net nocturnal CO_2 assimilation, diurnal fluctuations in organic acid accumulation (or titratable acidity), stomatal conductance, and abundant primary carboxylating (phosphoenolpyruvate carboxylase) or decarboxylating enzymes (e.g., NAD(P)-malic enzyme (ME), pyruvate orthophosphate dikinase (PPDK), phosphoenolpyruvate carboxykinase (PEPCK); Cockburn, 1985; Ting, 1985; Griffiths, 1988). Tracking these multiple parameters has led to the classification of various modes of CAM that serve to highlight the extreme plasticity of the CAM state.

In "obligate" or "constitutive" CAM species, net CO_2 uptake occurs almost exclusively at night even under well-watered conditions accompanied by large diel malate fluctuations. Other species that appear "nearly- C_3 " have been described as having a weak form of CAM termed "CAM cycling" displaying a C_3 gas exchange pattern along with diel fluctuations in C_4 acids, but little or no net nocturnal carbon assimilation

(Ting, 1985; Borland, 1996). In such species, recycling of respiratory CO_2 is thought to be the source of increased nocturnal titratable acidity (Winter *et al.*, 1986; Patel and Ting, 1987). The suggested value of CAM-cycling may include conservation of CO_2 (Martin and Zee, 1983) or water (Cockburn, 1985; Martin *et al.*, 1988; Borland, 1996), enhancement of water uptake (Lüttge, 1986, 1987; Harris and Martin, 1991), or minimization of photoinhibition (Osmond *et al.*, 1980; Osmond, 1982). Additionally, CAM-cycling plants may benefit from being poised to enter CAM rapidly when called for by prevailing environmental conditions (Sipes and Ting, 1985; Martin, 1996). A hypothetical variation of CAM cycling called “rapid-cycling CAM” has also been proposed in plants wherein both the CO_2 acquisition and reduction phases of CAM occur within time periods shorter than the normal diel cycle (Cockburn, 1998). Another permutation of CAM called “CAM-idling”, found typically in plants undergoing severe drought stress, is characterized by closure of stomata both day and night in the presence of continued diel fluctuations in organic acid concentrations. CAM-cycling and idling are thought to preserve and maintain the photosynthetic apparatus by limiting photoinhibition, maintaining a positive carbon balance during severe droughts, and allowing rapid recovery upon rehydration (Patel and Ting, 1987; Ting, 1985, 1987; Bastide *et al.*, 1993; Borland, 1996). Other modes of CAM, such as “latent CAM” wherein basal organic acid concentrations are elevated above those normally present in C_3 plants, but do not fluctuate diurnally (Schuber and Kluge, 1981), and may represent the nascent progression of C_3 to CAM in some species (Lee and Griffiths, 1987). The apparent continuum of CAM modes emanates from ontogenetic and environmental factors and the evolutionary history of the plant family (Borland and Griffiths, 1996).

17.4.2 ENVIRONMENT

Environmental stress induction of CAM has been reported for a variety of facultative or **C_3 /CAM-intermediate** genera. We will focus our discussion on *Mesembryanthemum* because physiological observations are well supported by molecular genetic data in this species (Edwards *et al.*, 1996; Cushman and Bohnert, 1996; Schmitt *et al.*, 1996). Stress factors, such as drought, salinity and high temperature, can result in rapid, reversible CAM induction in many species in which CAM is inducible. In comparison, changes in photoperiod initiate a slow, irreversible induction of CAM coordinated with development as has been well documented in *Kalanchoe* species (Brulfert *et al.*, 1982 a, b, 1988 a, b; Brulfert and Quieroz, 1982).

M. crystallinum exhibits rapid CAM induction in response to high salinity, drought, high light with low humidity, and exposure of roots to low temperature or anoxia (Winter and von Willert, 1972; Winter 1973 a, b; Winter, 1985). Induction is accompanied by increases in the activity of enzymes for malate formation/decarboxylation, glycolysis/gluconeogenesis, and starch synthesis/degradation (Holtum and Winter, 1982, Winter *et al.*, 1982; Paul *et al.*, 1993; Häusler *et al.*, 2000; see Table 1). Notably, the activities of C-6 compound processing enzymes are not induced (Holtum and Winter, 1982) and the activities of photorespiratory enzymes are not altered (Whitehouse *et al.*, 1991). Extended photoperiods accelerate the life cycle of the plant, stimulate malate accumulation, and induce the activity of NADP-ME and PEPC (Cheng and Edwards, 1991). Although plants exposed to constant illumination accumulated high amounts of malate and key CAM enzymes, diurnal fluctuations in

malate or titratable acidity were curtailed suggesting that CAM requires some dark period for the regulation of enzymes to commence. High light intensity and quality can act synergistically with NaCl or ABA to enhance the expression of the CAM-specific form of PEPC in *M. crystallinum* (McElwain *et al.*, 1992).

17.4.3 DEVELOPMENT

In *M. crystallinum* the capacity to perform CAM following environmental stress is influenced by leaf development (von Willert *et al.*, 1976 a, b; Ostrem *et al.*, 1987; Cushman *et al.*, 1990; Chu *et al.*, 1990; Herppich *et al.*, 1992; Bohnert *et al.*, 1994; Ratajczak *et al.*, 1994; Fißlthaler *et al.*, 1995). Increases in PEPC and PPDK expression or activity occur more slowly and to a lesser extent in younger plants than in older plants (Cushman *et al.*, 1990; Piepenbrock and Schmitt, 1991; Fißlthaler *et al.*, 1995). Increased PEPC activity and malate flux appears unlinked to the timing or magnitude of salt stress suggesting strictly ontogenetic control of the CAM induction process (Herppich *et al.*, 1992; Herppich and Herppich, 1997). The timing and extent of CAM induction or CAM-specific gene expression can vary considerably depending on photoperiod length, humidity, temperature, nutrient supply, or volume of the rooting medium (Cheng and Edwards, 1991; Schmitt and Piepenbrock 1992 a; Piepenbrock *et al.*, 1994). Reductions in cell and leaf turgor pressure and water content are observed in generally well watered plants and such decline progresses with age (Heun *et al.*, 1981; Rygol *et al.*, 1986; Winter and Gademann, 1991). This has led to the proposition that incremental development of low levels of CAM activity arises in response to mild water deficits rather than from a developmental program (Schmitt and Piepenbrock 1992 a; Winter and Gademann, 1991; Piepenbrock *et al.*, 1994). However, since the development of such a water deficit follows a developmental program, we seem to be left with a chicken-and-egg question that might be resolved by further experimentation. "Split root" experiments have shown that CAM induction can be triggered without disturbing leaf relative water content or mesophyll cell turgor. Whereas this experiment does not totally solve the dilemma, it suggests that roots perceive reductions in soil water availability, that a signal is generated at the root/soil interface and that this signal is conveyed to the leaves (Eastmond and Ross, 1997). Detached leaves, however, can initiate CAM induction (Schmitt, 1990; Dai *et al.*, 1994; Peters *et al.*, 1997; Taybi and Cushman, 1999) suggesting that root-derived signals are not necessary. Clearly, both developmental and environmental factors are involved in CAM induction with the environment probably making the largest contribution to this process.

17.4.4 SIGNAL TRANSDUCTION

Little is known about how environmental, hormonal, developmental, or tissue-specific inputs become integrated to induce or maintain CAM. Despite nearly a decade of descriptive research, the molecular mechanisms underlying these signaling pathways remain poorly understood. Preliminary studies suggest that CAM induction requires the coordination of a complex set of parallel, yet overlapping signal transduction pathways (Vernon *et al.*, 1993; Tsiantis, 1996).

17.4.4.1 Second messengers and plant growth regulators

In higher plants, intracellular Ca^{2+} acts as an essential second messenger in salinity and osmotic stress signaling pathways leading to the expression of genes encoding proteins involved in various adaptive or protective roles (Knight *et al.*, 1997, 1998). Recent studies in *M. crystallinum* confirm that intracellular Ca^{2+} also plays a key role in CAM induction. Detached leaves treated with the Ca^{2+} ionophore, ionomycin, and thapsigargin, a specific inhibitor of endomembrane Ca^{2+} -ATPases and stimulator of intracellular Ca^{2+} release, accumulate *Ppc1* transcripts in the absence of stress or ABA treatments (Tsiantis, 1996; Taybi and Cushman 1999). Similar results are observed for the V-ATPase subunit c (Tsiantis, 1996). In contrast, pretreatment with EGTA, an extracellular calcium chelator, completely abolished *Ppc1* mRNA increases following ABA and stress treatments (Taybi and Cushman, 1999). Increases in intracellular $[\text{Ca}^{2+}]$ derived from either extracellular or intracellular sources are thus likely to mediate stress signal transduction events leading to changes in gene expression preceding CAM induction.

Environmental stress also alters the amounts, relative proportions, and distribution of endogenous plant growth regulators implicating their involvement in signaling processes. The signals that are conveyed from root to leaves of *M. crystallinum* following the perception of water stress are unknown (Eastmond and Ross, 1997). Since cytokinin synthesis in roots is inhibited by water deficits (Hare *et al.*, 1997), it has been postulated that water stress limits the supply of cytokinin from the roots via the transpiration stream resulting in the de-repression of PEPC expression (Schmitt and Piepenbrock, 1992 b; Eastmond and Ross, 1997). Experimental treatment of detached leaves with cytokinins and the negative correlation of endogenous cytokinin levels during dehydration stress support this hypothesis (Schmitt and Piepenbrock, 1992 b; Dai *et al.*, 1994; Peters *et al.*, 1997). Like cytokinin, methyl jasmonate (MJA) inhibits PEPC transcript accumulation, enzyme activity and CAM induction in well-watered and salt-stressed *M. crystallinum* and accelerates the decline in PEPC transcript amounts in excised leaves undergoing stress recovery (Dai *et al.*, 1994; Schmitt *et al.*, 1996). Together, MJA and cytokinin (6-BAP) have an additive effect on the attenuation of PEPC and CAM induction. Consistent with a role in leaf senescence, a MJA-inducible and stress-inducible cysteine protease gene has been characterized in *M. crystallinum* that is expressed only in leaves (Forsthoefel *et al.*, 1998).

In contrast, salinity or drought stress induction of CAM is positively correlated with elevated endogenous abscisic acid (ABA) concentrations in root and leaf tissues in *Kalanchoë blossfeldiana* (Brulfert *et al.*, 1982 a; Taybi *et al.*, 1995), *Peperomia camptotricha* (Sipes and Ting, 1985), and *M. crystallinum* (Thomas *et al.*, 1992 b). Exogenous application of ABA also results in CAM induction (Ting, 1981, 1985; Taybi *et al.*, 1995), elevated CAM enzyme activities (Chu *et al.*, 1990; Dai *et al.*, 1994; Taybi *et al.*, 1995), and increased mRNA accumulation of CAM specific genes (Cushman *et al.*, 1993 a; Taybi *et al.*, 1995; Forsthoefel *et al.*, 1995 a, b; Tsiantis *et al.*, 1996). Farnesol, an analog of ABA and a potent antitranspirant, can also trigger CAM induction (Dai *et al.*, 1994). Furthermore, the efficacy of CAM induction by ABA can be influenced by light intensity (McElwain *et al.*, 1992). *Cis*-acting ABA-responsive elements (ABREs), present in the 5' flanking region of *Ppc1*, *GapC1*, and *Ppdk1* genes

may participate in altering CAM gene expression by ABA (Cushman and Bohnert, 1996; Schmitt *et al.*, 1996).

17.4.4.2 Protein kinases and phosphatases

The expression of various protein kinases and protein phosphatases is elevated following water stress implicating their participation in signal transduction cascades (Shinozaki and Yamaguchi-Shinozaki, 1996, 1997). Specific inhibitors of these signaling components can be used to measure the extent to which protein phosphorylation events mediate CAM induction. Pretreatment of detached *M. crystallinum* leaves with W7, a specific inhibitor of Ca^{2+} /calmodulin-dependent protein kinases, blocked *Ppc1* transcript accumulation in response to ionic, osmotic, and dehydration stress and ABA treatments (Taybi and Cushman, 1999) suggesting that Ca^{2+} /CaM protein kinases play a positive role in stress-induced increases in *Ppc1* expression. Pretreatment with okadaic acid, an inhibitor of protein phosphatase 2A and 1 activities, inhibited stress-responsive accumulation of *Ppc1* and *Imt1* transcripts, but not the induction of *Atpc1* (Tsiantis, 1996; Taybi and Cushman, 1999). Similar inhibition of *Ppc1* expression was also observed with cantharidic acid, a more specific inhibitor of protein phosphatase 2A, and lavendustin C (C5), a calmodulin kinase II inhibitor. While such results must be viewed with extreme caution due to possible nonspecific inhibition of other cellular processes, they implicate the participation of both protein kinases and phosphatases in stress signaling events.

To determine the functional contribution of specific protein kinases and phosphatases to CAM induction or maintenance requires the molecular cloning and characterization of candidate genes followed by disruption and/or overexpression studies. One Ser/Thr protein kinase (MK9) whose expression is induced following drought stress, ABA and 6-BAP treatments (J.C. Cushman, unpublished) has been characterized from *M. crystallinum* (Baur *et al.*, 1994) and may play a role in carbohydrate signaling. Additional protein kinases related to cyclic-nucleotide-dependent, Ca^{2+} -phospholipid-dependent kinases, and Ca^{2+} -dependent/calmodulin-like-protein kinases have also been isolated from *M. crystallinum* (Table 1, pp. 391-393) and may function downstream of cAMP, cGMP, or Ca^{2+} second messengers in signal transduction cascades. Another Ser/Thr kinase (MK10) contains conserved light-, oxygen-, or voltage sensing (LOV) motifs, named for environmental factors that can change redox status, that may function as a redox sensor in response to changes in light (Huala *et al.*, 1997). Alternatively, this protein kinase may function in signal transduction during the C_3 to CAM transition by sensing changes in the redox status of the electron transport chain known to occur in plants performing CAM (Krieger *et al.*, 1998).

Recently, more than 10 cDNA clones encoding protein phosphatase of the 2C family have been sequenced and characterized for their tissue- and environmental stress-specific expression patterns in *M. crystallinum* (Miyazaki *et al.*, 1999; Fukuhara T., personal communication). In Arabidopsis, (the ABI1 and ABI2 genes, which are involved in ABA signaling, are members of the PP2C family. Thus, it is expected that many PP2C proteins may also play roles in environmental stress signaling. The expression of one clone, designated MPC6, is high in juvenile leaf tissues, but gradually declines as the primary leaves age. This decline can be accelerated by salinity stress, low temperature and drought (Miyazaki *et al.*, 1999). This expression pattern is intriguing

since it suggests that MPC6 may be involved in negatively regulating the transition from C_3 to CAM. The absence of this phosphatase activity might lead to increased phosphorylation of another protein factor, such as a transcription factor triggering CAM-specific gene expression and ultimately CAM induction (Cushman and Bohnert, 1992). While this correlation is not a proof, this hypothesis can be easily tested experimentally, for example, by transgenic ectopic antisense expression of this PP2C.

17.5 Molecular genetics of CAM

A full understanding of the molecular genetic basis of CAM and dissection of the molecular mechanisms underlying the recruitment and regulation of CAM-specific genes and their gene products requires a significant compilation of gene sequences and predicted protein sequences. Efforts by several laboratories have resulted in the characterization of genes and their regulatory properties associated with various aspects of CAM, predominantly from one species, *M. crystallinum* (see Table 1, pp.391-393). Genes encoding enzymes essential for diurnal carbon flux during CAM (e.g. carboxylases/decarboxylases, glycolytic/gluconeogenic and starch forming and degrading enzymes) have been characterized (Table 1). For example, in only a few species such as *M. crystallinum*, *K. blossfeldiana*, and *Vanilla planifolia* have specific PEPC gene family members been identified that are selectively recruited for CAM-specific roles (Rickers *et al.*, 1989; Cushman *et al.*, 1989; Gehrig *et al.*, 1995; Honda *et al.*, 1996; Gehrig *et al.*, 1999). For other enzymes, at least one CAM-specific isogene from multigene families has been reported with most work being done in *M. crystallinum* (Table 1). However, large gaps exist in our knowledge of other enzymes that are localized to distinct subcellular compartments such as mitochondrial NAD-malic enzyme and cytosolic NADP-malic enzyme (Holtum and Winter, 1982; Cushman, 1992; Saitou *et al.*, 1994; Cook *et al.*, 1995) or cytosolic NAD-malate dehydrogenase and chloroplastic NADP-malate dehydrogenase (Holtum and Winter, 1982; Cushman, 1993; Saitou *et al.*, 1995; Ocheretina and Scheibe, 1997). Dramatic changes occur during the shift from C_3 photosynthesis to CAM in the activities of starch degrading enzymes (Paul *et al.*, 1993) and profiles of starch degradation products exported from chloroplasts (Neuhaus and Schulte, 1996). In particular, chloroplastic glucan phosphorylase and glucan hydrolyases activities increase following CAM induction (Häusler *et al.*, 2000). Yet little is known about the underlying molecular genetic mechanisms responsible for these changes.

17.5.1 LARGE-SCALE GENE CHARACTERIZATION

Only about 1% of all genes has been characterized from *M. crystallinum*. To sort out the functional contribution of enzymes, transporters, and regulatory factors controlling the CAM pathway, a comprehensive characterization of their genes or gene families is needed. One approach for rapidly accumulating molecular genetic information is to conduct large-scale sequencing of anonymous cDNAs or expressed sequence tags (ESTs). Several EST projects have been initiated in *M. crystallinum* targeting different tissues (e.g., leaves, roots, epidermal bladder cells) from well-watered plants performing C_3 photosynthesis and salinity- or drought-stressed plants performing CAM (Cushman

et al., 1999 b; D.E. Nelson and H.J. Bohnert, unpublished). ESTs provide an invaluable resource that complements genetic analyses and provide markers for genome mapping and sequencing and large-scale gene expression studies in normal, mutant, and transgenic plants created using reverse genetic approaches (Newman *et al.*, 1994; Cooke *et al.*, 1996; Delseny *et al.*, 1997). Undertaking large-scale random sequencing of cDNAs in a CAM species is well justified in light of the large numbers of CAM species, estimated to be about 6% of all higher plant species, and their importance to xerophytic and epiphytic ecosystems (Smith and Winter, 1996; Zotz and Ziegler, 1997). An additional incentive for conducting EST analyses of CAM plants is that they may serve as rich sources of novel genes. For example, simultaneous EST sequencing of leaf cDNAs derived from well watered and salinity stressed *M. crystallinum* has revealed that stress plants contain approximately 15% more uncharacterized or novel transcripts than do well watered plants. Assuming that about 6000 genes are leaf-specific (Goldberg, 1988) and assuming that root tissues contain a comparable number of stress-specific genes, it can be argued that approximately 1,000-2,000 stress-related genes are underrepresented or missing from EST sequencing efforts surveying genes from unstressed plants. The number of novel stress-inducible genes present in halophytes may in fact be higher than this. A well-documented example is the extension of the inositol biosynthesis pathway by IMT1 (inositol O-methyltransferase), the enzyme that leads to the accumulation of methylated inositols in *M. crystallinum*, which seems to be absent from many other species (Vernon and Bohnert, 1992; Ishitani *et al.*, 1996). Many CAM plants are found in families and orders with only a few agronomically relevant species, and most are found in extreme habitats to which they have adapted over approximately 150 million years (Raven and Spicer, 1996). CAM species in these families are likely sources of useful regulatory and coding regions absent in many C_3 or C_4 crop species.

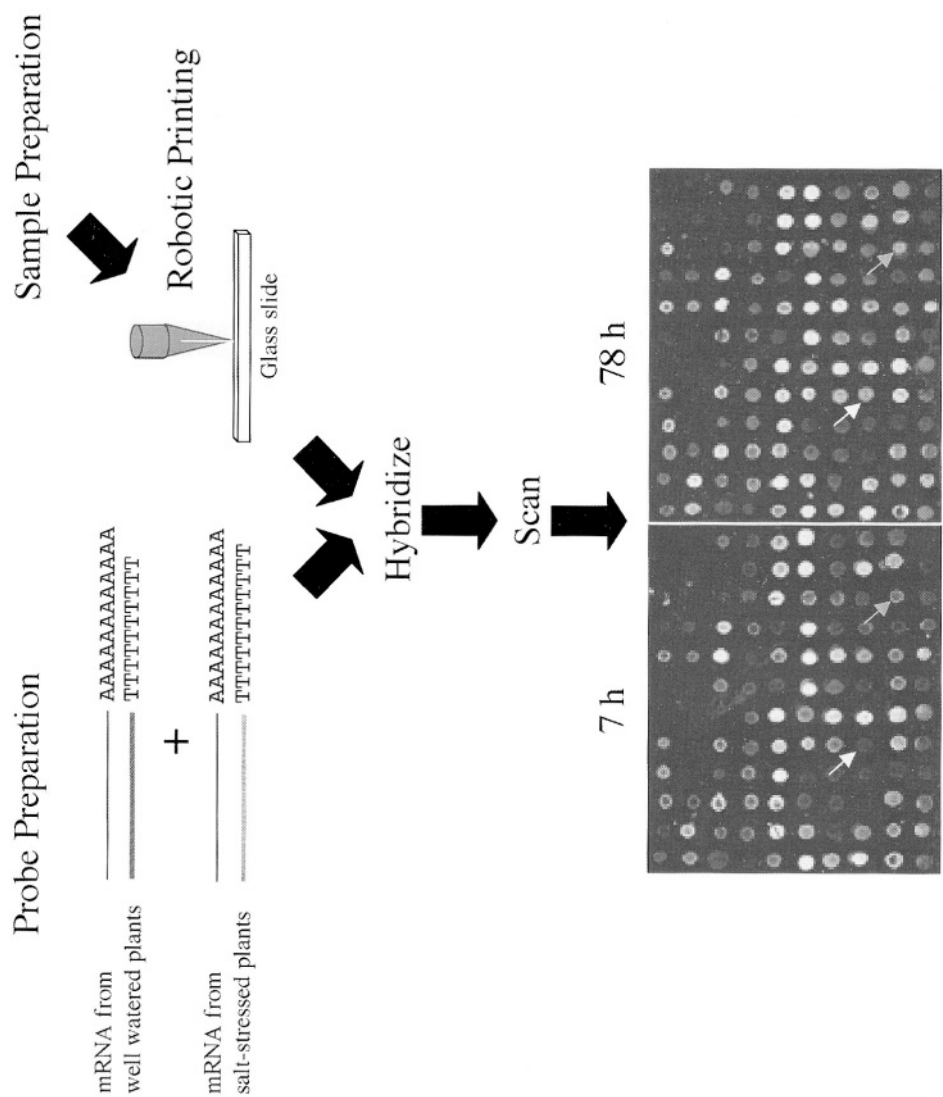
17.5.2 LARGE-SCALE GENE EXPRESSION PROFILING

The availability of large EST collections will also be essential for monitoring large-scale gene expression changes using microarray or DNA chip technology (Schena, 1996; Schena *et al.*, 1998; Lemieux *et al.*, 1998). Microarray profiling provides detailed information about the expression patterns of large numbers of genes and often provides the first functional information about unidentified genes (Figure 2). The greatest advantage, however, is that the printed arrays can monitor thousands of elements, each representing a coding region or 3' UTR region simultaneously. In principle, we can now consider monitoring gene expression changes not in weeks, days or hours, but in minutes through the entire life cycle of a plant. In Arabidopsis, microarray approaches have been used to assess mRNA accumulation profiles in specific tissues or organs (Schena *et al.*, 1995; Ruan *et al.*, 1998). This technology also offers a powerful tool to conduct integrative analysis of the effects of salinity stress or other environmental factors on changes in gene expression (Kehoe *et al.*, 1999). It will be particularly useful in gathering clues about the possible function of unknown genes by determining their tissue-specific, or even cell-specific expression patterns over a wide range of developmental phases and environmental stress conditions, high salinity being just one of many factors of agronomical importance that will be monitored. DNA microarray technology will also be an important tool for rapidly establishing 'expression fingerprints' in plant populations of, for example, mutants or transgenic, near-isogenic

and substitution lines, to identify gene collections associated with specific biological processes (Kehoe *et al.*, 1999). We have begun to use *M. crystallinum* EST clones to analyze genes that are induced or repressed by salinity stress (Figure 2). Once a gene expression profile of interest is identified for a particular EST, the clone can be sequenced and the full-length clone isolated and characterized further. Specific hypotheses about the function of selected genes can be tested by over- or under-expressing each gene in transgenic plants or by "knocking out" the gene using random or targeted insertional mutagenesis procedures. The phenotype of such specifically designed mutants can then be monitored in plants under a variety of stress conditions. Additional detailed information about temporal and spatial expression patterns and subcellular localization of a particular gene can also be obtained by expression of promoter-reporter gene or protein-reporter fusions in transgenic plants. Ultimately, DNA microarrays will permit genome-wide monitoring of gene expression of the effects of the loss or over- or wider-expression of one gene on the expression of other genes. Such monitoring will also improve our understanding of the complex regulatory circuits that govern interactions between the developmental and environmental stimuli leading to CAM induction.

17.6 Modulation of Gene Expression

The stress-induced switch from C_3 photosynthesis to CAM serves as an important paradigm for understanding how a multitude of gene expression and regulatory changes become integrated to bring about a new metabolic equilibrium to maintain this complex adaptation to environmental stress. A concerted series of transcriptional, post-transcriptional, and translational controls bring about the requisite build-up of CAM-related enzyme activities during this transition (Holtum and Winter, 1982; Winter *et al.*, 1982; Paul *et al.*, 1993). Most, but not all, of these changes correlate with increases in enzyme protein amounts (Winter *et al.*, 1982; Michalowski *et al.* 1989; Schmitt *et al.*, 1989) arising from *de novo* protein synthesis (Foster *et al.*, 1982; Höfner *et al.*, 1987). In addition, tonoplast H^+ -translocating ATPase (V-ATPase) and Na^+/H^+ transport activities are enhanced during the CAM switch (Vera-Estrella *et al.*, 1999; Barkla *et al.*, 1999) as a result of increased expression of V-ATPase subunits (Lüttge *et al.*, 1995; Dietz and Arbingner, 1996; Löw *et al.*, 1996; Tsiantis *et al.*, 1996; Chapter 19). Light-dependent chloroplast pyruvate transport (Kore-eda *et al.*, 1996) and, presumably, triose and hexose phosphate transporters are also induced or enhanced during CAM induction (Neuhaus and Schulte, 1996). Häusler *et al.* (2000) have recently shown that CAM induction results in a dramatic induction of transcripts encoding a phosphoenolpyruvate (PEP) phosphate translocator (PPT) and a glucose-6-phosphate (Glc6P) phosphate translocator (GPT) with expression peaking in the light period, whereas transcripts for a chloroplast glucose transporter (pGlcT) and a triose phosphate transporter (TP) remain largely unchanged. These results coupled with metabolite, enzyme activity, and transport measurements demonstrate that Glc6P is the main substrate for daytime starch biosynthesis during CAM.



17.6.1 TRANSCRIPTIONAL REGULATION

What controls the molecular genetic basis of CAM induction. We have shown that transcriptional activation of CAM-specific genes is regulated primarily at the level of transcription and is mediated by the action of transcriptional activator and repressor proteins through interactions in the promoter regions of these genes. This was demonstrated directly using run-off transcription assays with nuclei isolated from leaves of *M. crystallinum* (Figure 3). Transcription rates rise from two- to six-fold in response to salinity stress depending on the gene in question (Cushman *et al.*, 1989; Cushman, 1992, 1993; Vernon *et al.*, 1993; Forsthoefel *et al.*, 1995 a, b). As a consequence, mRNA accumulation for CAM-specific genes such as *Ppc1* can increase within 2-3 hours following salinity or dehydration stress of detached leaves of *M. crystallinum* (Schmitt, 1990) or *K. blossfeldiana* (Brulfert *et al.*, 1993; Taybi *et al.*, 1995). Transcriptional activation events were also confirmed using promoter-reporter gene fusions in a transient expression system based on microprojectile bombardment of detached *M. crystallinum* leaves (Schaeffer *et al.*, 1995). The system was used to identify *cis*-acting enhancer and silencer regulatory regions present in the *Ppc1* and *GapC1* promoters and to identify regions sufficient for salt-inducible gene expression (J. C. Cushman, unpublished). However, additional *cis*-acting elements for light- and ABA-responsive gene expression and putative binding sites for a variety of DNA binding proteins are present in the 5' flanking regions of stress-inducible genes such as *Ppc1*, *Ppdk1*, and *GapC1* (Cushman *et al.*, 1993a; Schmitt *et al.*, 1996). Multiple DNA-binding proteins interact with the *Ppc1* and *GapC1* 5' flanking region that show increased abundance or DNA-binding affinity following salt stress (Cushman and Bohnert, 1992; Schaeffer *et al.*, 1995). When expressed in transgenic tobacco, the *Ppc1* promoter fails to direct salt inducible gene expression suggesting that tobacco lacks the requisite transcriptional machinery responsible for recognizing the specific salt responsive promoter elements present in *M. crystallinum* (Cushman *et al.*, 1993 b). Based on these limited analyses, the regulatory mechanisms that mediate transcriptional activation of CAM-specific genes remain unclear, however, more detailed work characterizing *cis*- and *trans*-acting elements should improve our understanding of transcriptional activation events responsible for CAM induction and maintenance.

Figure 2. Microarray analysis of randomly selected cDNA from well watered and salinity stressed *M. crystallinum*. cDNA inserts were prepared by amplification using the polymerase chain reaction (PCR) and the DNA was spotted onto silylated glass slides using a robotic spotting device. Slides were then hybridized with a mixture of fluorescently labeled first-strand cDNA synthesis probes leaf RNA isolated from well-watered plants (Red, Cy5-dCTP) or from plants that had been NaCl-stressed (Green, Cy3-dCTP) for 7 h and 78 h, respectively. Following hybridization, the slides were washed to remove residual dyes and scanned sequentially using a confocal dual laser scanning device outfitted with a photomultiplier tube to capture fluorescent dye emissions. A composite image of two false color images is constructed to show the expression patterns detected by each probe. The array shown contains 120 cDNA inserts that had been selected at random from a leaf cDNA library generated from well watered (top six rows) or stressed (30 h with 0.5 M NaCl, bottom four rows) *M. crystallinum*. Genes strongly expressed in unstressed plants appear in red, whereas those expressed under both well watered and stressed conditions appear in yellow. Arrows indicate strongly NaCl-stress-induced cDNAs (green): white arrow = *Int1*, purple arrow = *Sep1* (see color version in the color section).

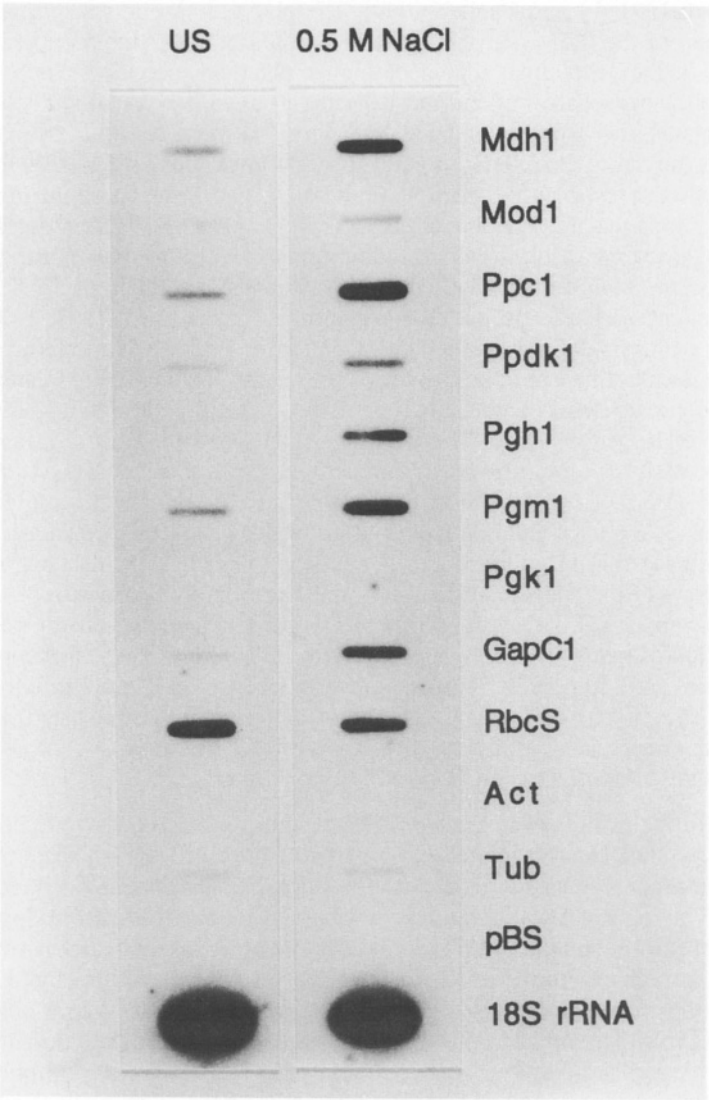
17.6.2 POSTTRANSCRIPTIONAL AND TRANSLATIONAL REGULATION

Although transcriptional induction and changes in mRNA populations following stress are well-documented (Ostrem *et al.*, 1987), changes in mRNA utilization and translational efficiency are also likely to govern gene expression changes during the C₃ to CAM transition. Comparisons of transcription rates and transcript accumulation patterns in *M. crystallinum* undergoing CAM induction at different ages have revealed that *Ppc1* mRNA stability may be enhanced in older plants (Cushman *et al.*, 1990). In contrast, *RbcS* transcripts encoding the small subunit of Rubisco decline rapidly upon salt stress (DeRocher and Bohnert, 1993; Bohnert *et al.*, 1999). An increase in chloroplast RNA-binding proteins accompanies CAM induction (Breiteneder *et al.*, 1994). Such proteins may act to stabilize specific transcripts. Also, transcription rates and mRNA accumulation for enolase increase in response to salinity stress occur without a corresponding increase in protein amounts (Forsthoefel *et al.*, 1995 a) suggesting that translational control mechanisms modulate the expression of this and perhaps other glycolytic enzymes. During the transition from C₃ to CAM, total protein synthesis declines, whereas the synthesis of specific proteins, such as PEPC, increases (Höfner *et al.*, 1987). Altered translational activity is correlated with changes in mRNA distribution on polysomes (H.J. Bohnert, unpublished) and enhanced expression of a ribosome-inactivating protein (RIP) in *M. crystallinum* that becomes up-regulated following CAM induction with a dampened diurnal expression pattern (Rippmann *et al.*, 1997). Activity of this RIP has been documented and it may alter translation profiles through the selective turnover of ribosomes in response to stress. Alterations in the expression of specific stress-induced proteins, such as proteases, may also bring about rapid and selective turnover of proteins to fuel *de novo* protein synthesis (Forsthoefel *et al.*, 1998). At present, support for posttranscriptional and translational control mechanisms are largely correlative. Thus, future efforts directed at elucidating the contribution of these mechanisms in controlling CAM induction processes are needed.

17.6.3 POSTTRANSLATIONAL REGULATION

Posttranslational control mechanisms regulate diurnal or circadian activities of several key CAM enzymes. Most studies have focused on the reversible day/night phosphorylation of PEPC by PEPC kinase in *Kalanchoë fedtschenkoi*, which tightly controls PEPC activity to avoid futile cycles of carboxylation and decarboxylation

Figure 3. In vitro transcription run-on assays of CAM specific genes. Cloned gene-specific probes for enzymes involved in malate metabolism and glycolysis/gluconeogenesis were blotted onto nitrocellulose and hybridized with transcripts radiolabeled *in vitro* by isolated nuclei from leaves of plants that were well watered (unstressed, US) or stressed for 5 days with 0.5 M NaCl (0.5 M NaCl). Transcription rates increased anywhere from 2- to 5-fold for: *Mdh1*, NADP⁺ malate dehydrogenase; *Mod1*, NAD(P)⁺ malic enzyme; *Ppc1*, phosphoenolpyruvate carboxylase; *Ppd1*, Pyruvate orthophosphate dikinase; *Pgh1*, Enolase; *Pgm1*, phosphoglycerate mutase; *Pgk1*, phosphoglycerate kinase; *GapC1*, glyceraldehyde 3-phosphate dehydrogenase. Those genes not induced by salt stress include: *rbcS*, small subunit of ribulose-1,5-bisphosphate carboxylase/ oxygenase; *Act*, actin, *Tub*, tubulin, 18S rRNA, 18S ribosomal RNA from soybean. Negative control, pBS, Bluescript SK⁺ (from Cushman *et al.*, 2000).



(Nimmo *et al.*, 1984; Nimmo *et al.*, 1986; Brulfert *et al.*, 1986; Baur *et al.*, 1992; Weigend, 1994; Li and Chollet, 1994). The phosphorylation site responsible for night activation of the CAM enzyme has been located at a highly conserved serine residue present in the N-terminal region of higher plant polypeptides which is absent from bacterial or cyanobacterial enzymes (Chollet *et al.*, 1996; Vidal and Chollet, 1997). In CAM plants, the dephosphorylated "day form" is more sensitive to malate inhibition, whereas the more active, phosphorylated "night form" has higher affinity for PEP and is more sensitive to Glucose-6-phosphate and triose phosphate (positive effectors), but less sensitive to L-malate (negative effector) (Nimmo *et al.*, 1987; Jiao and Chollet, 1991). PEPC kinase expression is induced coincidentally with its target substrate by salt stress in *M. crystallinum* (Li and Chollet, 1994). Circadian regulation of PEPC kinase activity is dependent upon mRNA and protein synthesis (Carter *et al.*, 1991, 1996). In contrast, protein synthesis-dependent dephosphorylation of PEPC by a protein phosphatase 2A (PP2A) does not appear to be regulated by an endogenous rhythm (Carter *et al.*, 1990). PEPC kinase activity is regulated primarily by controlling the amounts of its translatable mRNA in *K. fedtschenkoi* (Hartwell *et al.*, 1996) with PEPC kinase mRNA being approximately 20 times more abundant at night than during the day. Activity disappears within 3 hours of the decline in translatable mRNA indicating rapid protein turnover (Hartwell *et al.*, 1996). Inhibition of translation also blocks the appearance of translatable PEPC kinase mRNA indicating that upstream signaling events controlling circadian rhythmicity of the enzyme also require protein synthesis (Hartwell *et al.*, 1996). These observations confirm that transcriptional regulatory events can not only govern the production of many CAM enzymes, but also control their post-translational regulation. The genes encoding PEPC kinase have now been cloned and partially characterized from a variety of CAM species providing direct confirmation that the expression of PEPC kinase is induced by salinity stress in *M. crystallinum* and is controlled by circadian oscillator (Hartwell *et al.*, 1999; Taybi *et al.*, 2000).

In addition to PEPC, several other CAM enzymes undergo post-translational regulatory phosphorylation events. In PEPCK-type CAM plants, PEPCK is phosphorylated at night and dephosphorylated during the day in *Tillandsia fasciculata* (Walker and Leegood, 1996). This phosphorylation pattern is likely to modulate decarboxylase activity over the course of the diurnal cycle to avoid futile carboxylation cycles between PEPC and PEPCK as both enzymes are localized to the cytosol. However, it is not known how this occurs or whether PEPCK is regulated by light or in response to a circadian rhythm. Covalent modification of NAD-ME may also be responsible for diurnal changes in the kinetic properties of this enzyme (Cook *et al.*, 1995). Post-transcriptional control of enolase activity by reversible phosphorylation has been suggested for enolase from *M. crystallinum* (Forsthoefel *et al.*, 1995 a). Covalent modification of many chloroplastic and possibly cytosolic enzymes such as NAD-GAPDH, NADP-MDH, FBP, and enolase via the covalent modification of redox-sensitive thiol groups provide yet another example of posttranslational control for the reversible activation of CAM enzymes (Anderson *et al.*, 1995, 1998).

17.7 Future Perspectives

Ten years ago, the molecular analysis of the C_3 to CAM switch began with the demonstration in *M. crystallinum* that PEPC was transcriptionally induced, and that one isoform of the PEPC gene family was responsible for the increased protein amount (Rickers *et al.*, 1989; Cushman *et al.*, 1989). In years since, many discoveries shed light not only on the induction of CAM, the genes for CAM enzymes and their expression in C_3 and CAM mode, but also on the complex machinery with which *M. crystallinum* can survive traumatic environmental insults.

Which are the challenges that lie ahead for our understanding of either CAM or stress tolerance? Foremost, is the need for a tractable genetic system for studying CAM. In this regard, a concerted effort by several laboratories to generate, characterize, and maintain CAM mutant populations should provide an important resource to address future questions in CAM molecular genetics. Mutant collections have already been generated in *M. crystallinum* using irradiation with fast neutrons and gamma rays or treatment with ethylmethane sulfonate (EMS) (Adams *et al.*, 1998). Advances in the transformation and regeneration efficiency of *M. crystallinum* are also needed to improve the ease of conducting reverse genetic screens and testing the function of specific genes by silencing, adding, or ectopically expressing genes of interest. For example, the identification of mutants or targeted elimination of specific genes to produce a stress-sensitive phenotype will be essential to elucidate the genetic basis of essential, sufficient, or ancillary tolerance mechanisms.

Another challenge will be the sequencing and annotating the complete genome of *M. crystallinum*. Only a few years ago, this suggestion would have been met with ridicule, however, the rapid technological advances that have been made in DNA sequencing and bioinformatic resources have brought this goal within reach. The first step towards this goal, the partial sequencing of large numbers of cDNA in order to catalogue the expressed genes (ESTs) of *M. crystallinum*, is well underway with special emphasis being placed on those genes expressed in stressed plants. Although it is impossible to predict the exact number of ‘stress-specific’ genes, we estimate that approximately 10% of the ice plant genome, or about 2,000 genes, will be preferentially expressed in stressed plants. Among these, we expect some novel genes, specific to the ice plant, or genes unique for the Aizoaceae family, or the order Caryophyllales. Such novel genes might be responsible for structural or regulatory alterations in ion uptake, exclusion or partitioning mechanisms that distinguish halophytic plants from glycophytes. Other novel genes, as described earlier, will likely be involved in carbohydrate biosynthesis, carbon flux and accumulation - the accumulation of methylated inositols being one example (Adams *et al.*, 1992; Vernon and Bohnert, 1992).

Still other genes can be expected to participate in novel signal transduction and transcriptional activation or repression events. This assumption is based on obvious differences between plants performing C_3 or C_4 photosynthesis and CAM plants or between glycophytes and halophytes. For example, glycophytes seem to lack certain proteins that activate ice plant promoters. The IMT1 promoter and the promoter for the CAM-specific PEPC enzyme, for example, are strongly upregulated in *M. crystallinum* after stress. After transfer into tobacco, however, these promoters fail to direct salinity-

induced gene expression suggesting that *M. crystallinum* has evolved a unique set of regulatory elements and cognate transcription factors that are absent from tobacco and possibly other species. This observation is surprising, since many promoters are exchangeable across the monocot-dicot boundary. Such differences will have important implications for future biotechnology applications of transgene expression systems expressing novel genes conferring stress-adaptive traits. Unique sets of cognate promoter elements/transcription factors could prove useful for genetic engineering purposes by directing ectopic, stress-inducible gene expression programs having stress-adaptive value without undue influence on other essential metabolic processes.

Based on its past impact on studies involving CAM induction and salinity stress tolerance, we are confident that *M. crystallinum* will continue to have a major influence on plant biology for several reasons. First, it will continue to serve as an important source of interesting, novel, and useful enzymes and regulatory factors for genetic engineering strategies aimed at improving the competitiveness and survival of crop plants in xeric or saline aquatic habitats targeting improved salinity and drought tolerance. Second, it will serve a useful model for developmental plant biology especially since a detailed outline of its growth patterns and life cycle of the plant has been established under unstressed and stressed conditions (Adams *et al.*, 1998). Using well-defined, highly reproducible conditions, it should be possible to piece together the puzzle of multi-faceted interactions between developmental and environmental stimuli. Finally, research on *M. crystallinum* will continue to contribute to our knowledge of the ways in which environmental stress is sensed and converted into stress-responsive adaptations.

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TABLE 1. Enzymes and genes involved in Crassulacean Acid Metabolism. Dashes (-) indicate that the genes are not induced. Question marks (?) indicate that the gene, its expression pattern, or the subcellular localization of the gene product has not been described in a CAM species. Intracellular localization of gene products: L, CYT, cytoplasm; CP, chloroplast; MT, mitochondria; PER, peroxisome; TP, tonoplast; NC, nucleus; VAC, vacuole.

Enzyme	Gene	Source Organism	Sub-cellular Location	Expression Pattern (tissue/inducers)	Reference
Carboxylases/Decarboxylases:					
Phosphoenolpyruvate carboxylase	<i>Ppc1</i>	<i>M. crystallinum</i>	CYT	Leaf/NaCl, ABA, drought, BAP, light	Chu et al., 1990; Cushman et al., 1989; McElwain et al., 1982; Rickers et al., 1989; Thomas et al., 1992; Thomas and Bohnert, 1993
	<i>Ppc2</i>	<i>M. crystallinum</i>	CYT	Leaf, root/-	Cushman and Bohnert, 1989 b
	<i>Ppc1,2</i>	<i>Kalanchoe blossfeldiana</i>	CYT	Leaf/ABA, drought, short day length	Gehrig et al., 1995; Taybi et al., 1995
	<i>Ppc3,4</i>	<i>K. blossfeldiana</i>	CYT	??	Gehrig et al., 1995; Taybi et al., 1995
	<i>Ppc1</i>	<i>Vanilla planifolia</i>	CYT	Leaf, stem/?	Gehrig et al., 1999
	<i>Ppc2</i>	<i>V. planifolia</i>	CYT	Root/?	Gehrig et al., 1999
	<i>Ppc1</i>	<i>Aloe arborescens</i>	CYT	Green leaf/?	Honda et al., 1996
Decarboxylases:					
NADP-Malic enzyme	<i>Mod1</i>	<i>M. crystallinum</i>	CYT	Leaf/NaCl	Cushman, 1992
NAD-Malic enzyme	?	<i>M. crystallinum</i>	MT	Leaf/NaCl	Holtum and Winter, 1982
PEP carboxykinase	<i>Ppck1</i>	<i>Tillandsia fasciculata</i>	CYT	??	Walker and Leegood, 1996; Gehrig et al., unpublished
		<i>T. utriculata</i>			
		<i>Nidularium fulgens</i>			
		<i>Aloe aristata</i>			
		<i>Hoya carnosa</i>			
		<i>M. crystallinum</i>			
Malate metabolism enzymes:					
NADP-Malate dehydrogenase	<i>Mdh1</i>	<i>M. crystallinum</i>	CP	Leaf/NaCl	Cushman, 1993
NAD-Malate dehydrogenase	<i>Mdh2</i>	<i>M. crystallinum</i>	CYT	Leaf/NaCl	Ocheretina and Scheibe, 1997
Glycolytic/gluconeogenic enzymes:					
Pyruvate orthophosphate dikinase	<i>Ppdk1</i>	<i>M. crystallinum</i>	CP	Leaf/NaCl	Fisslthaler et al., 1995
Enolase	<i>Pgh1</i> ; <i>l...2</i>	<i>M. crystallinum</i>	CYT	Leaf, root/NaCl, drought, cold, hypoxia, ABA, BAP	Forsthoefel et al., 1995 a

Enzyme	Gene	Source Organism	Sub-cellular Location	Expression Pattern (tissue/inducers)	Reference
Phosphoglyceromutase	<i>Pgm1</i>	<i>M. crystallinum</i>	CYT	Leaf, root/NaCl, drought, ABA, BAP	Forsthoefel <i>et al.</i> , 1995 b
Phosphoglycerate kinase	<i>Pgk1</i>	<i>M. crystallinum</i>	CYT	NaCl	J.C. Cushman, unpublished
Phosphoglycerate kinase	?	<i>M. crystallinum</i>	CP	NaCl	Winter <i>et al.</i> , 1982
NAD-Glyceraldehyde 3-phosphate dehydrogenase	<i>GapC1</i>	<i>M. crystallinum</i>	CYT	NaCl	Ostrem <i>et al.</i> , 1990
NADP- Glyceraldehyde 3-phosphate dehydrogenase	?	<i>M. crystallinum</i>	CP	NaCl	Holtum and Winter, 1982
Phosphoglucumutase	<i>Pgl1</i>	<i>M. crystallinum</i>	CYT	?	H.J. Bohnert, unpublished
Fructose 1,6-bisphosphatase	<i>Fbp1</i>	<i>M. crystallinum</i>	CP	NaCl	Holtum and Winter, 1982; J.C. Cushman, unpublished
Phosphofructokinase	<i>Pfk1</i>	<i>M. crystallinum</i>	CP	?	Holtum and Winter, 1982; J.C. Cushman, unpublished
Pyruvate kinase	<i>Pyk1</i>	<i>M. crystallinum</i>	?	?	Holtum and Winter, 1982; J.C. Cushman, unpublished
Hexokinase	<i>Hek1</i>	<i>M. crystallinum</i>	CYT	NaCl	J.C. Cushman, unpublished
Fructose-bisphosphatase aldolase	<i>Fba1</i>	<i>M. crystallinum</i>	CYT	NaCl	J.C. Cushman, unpublished
Glucose-6-phosphate dehydrogenase	<i>Gpd1</i>	<i>M. crystallinum</i>	?	?	Holtum and Winter, 1982; J.C. Cushman, unpublished
Phosphohexose isomerase	<i>Gpi1</i>	<i>M. crystallinum</i>	CYT/CP	NaCl	Holtum and Winter, 1982; J.C. Cushman, unpublished
Alpha-amylase	?	<i>M. crystallinum</i>	CP/CYT?	NaCl	Paul <i>et al.</i> , 1993
Beta-amylase	?	<i>M. crystallinum</i>	CYT?	NaCl	Paul <i>et al.</i> , 1993
D-enzyme	?	<i>M. crystallinum</i>	CP/CYT?	NaCl	Paul <i>et al.</i> , 1993
R-enzyme	?	<i>M. crystallinum</i>	CP/CYT?	NaCl	Paul <i>et al.</i> , 1993
Starch phosphorylase	<i>Pho1</i>	<i>M. crystallinum</i>	CP	NaCl, diurnal rhythm	Paul <i>et al.</i> , 1993; Häusler <i>et al.</i> , 2000; J.C. Cushman, unpublished
Plastidic transporters:					
Phosphoenolpyruvate phosphate translocator	<i>Ppt1</i>	<i>M. crystallinum</i>	CP	NaCl, diurnal rhythm	Häusler <i>et al.</i> , 2000
Glucose-6-phosphate phosphate translocator	<i>Gpt1</i>	<i>M. crystallinum</i>	CP	NaCl, diurnal rhythm	Häusler <i>et al.</i> , 2000
Triose phosphate phosphate translocator	<i>Tpt1</i>	<i>M. crystallinum</i>	CP	-	Häusler <i>et al.</i> , 2000

Enzyme	Gene	Source Organism	Sub-cellular Location	Expression Pattern (tissue/inducers)	Reference
Photorespiration enzymes					
Glycolate oxidase	<i>Glx1</i>	<i>M. crystallinum</i>	PER	?	H.J. Bohnert, unpublished
Serine hydroxymethyl transferase	<i>Sht1</i>	<i>M. crystallinum</i>	MT	-	J.C. Cushman, unpublished
Serine aminotransferase	<i>Sat1</i>	<i>M. crystallinum</i>	PER	-	J.C. Cushman, unpublished
Glycine cleavage protein H	<i>GcpH1</i>	<i>M. crystallinum</i>	PER	?	H.J. Bohnert, unpublished
Glycine cleavage protein T	<i>GcpT1</i>	<i>M. crystallinum</i>	PER	?	H.J. Bohnert, unpublished
Protein kinases:					
PEPC kinase	<i>Ppck1</i> <i>2</i>	<i>K. fedtschenkoi</i>	CYT	Leaf/NaCl, diurnal rhythm	Hartwell <i>et al.</i> , 1999; Taybi <i>et al.</i> , 2000
SNF1 kinase	<i>MK1</i>	<i>M. crystallinum</i>	CYT	?	J.C. Cushman, unpublished
SNF1 kinase	<i>MK9</i>	<i>M. crystallinum</i>	CYT	leaf/NaCl, drought, ABA, 6-BAP	Baur <i>et al.</i> , 1994; J.C. Cushman, unpublished
AGC kinase	<i>MK6</i>	<i>M. crystallinum</i>	CYT	Leaf/NaCl, diurnal rhythm	B. Baur, unpublished
AGC kinase	<i>MK10</i>	<i>M. crystallinum</i>	CYT	?/redox sensing?	B. Baur, unpublished
Ca ²⁺ -dependent protein kinase	<i>CPK1,2</i>	<i>M. crystallinum</i>	CYT	Leaf, root/NaCl, drought	Patharkar and Cushman, 2000; J.C. Cushman, unpublished
Protein phosphatases					
Protein phosphatase 2C	<i>Mpc6</i>	<i>M. crystallinum</i>	CYT	young leaf, root/*repressed by NaCl, drought, cold	Miyazaki <i>et al.</i> , 1999
Tonoplast enzymes					
H ⁺ -ATPase, A subunit	<i>Atpv4</i>	<i>M. crystallinum</i>	TP	Leaf, root/NaCl	Löw <i>et al.</i> , 1996
H ⁺ -ATPase, B subunit	<i>AtpvB</i>	<i>M. crystallinum</i>	TP	Leaf, root/NaCl	Löw <i>et al.</i> , 1996
H ⁺ -ATPase, E subunit	<i>AtpvE</i>	<i>M. crystallinum</i>	TP	Leaf, root/NaCl	Dietz and Arbing, 1996
H ⁺ -ATPase, F subunit	<i>AtpvF</i>	<i>M. crystallinum</i>	TP	Leaf, root/NaCl	J.C. Cushman, unpublished
H ⁺ -ATPase, G subunit	<i>AtpvG</i>	<i>M. crystallinum</i>	TP	Leaf, root/NaCl	J.C. Cushman, unpublished
H ⁺ -ATPase, c subunit	<i>AtpvC</i>	<i>M. crystallinum</i>	TP	Leaf, root/NaCl, ABA, light	Bartholomew <i>et al.</i> , 1996; Löw <i>et al.</i> , 1996; Tsiantis <i>et al.</i> , 1996
H ⁺ -ATPase, 54kDa subunit	<i>Atpv</i>	<i>K. daigremontiana</i>	TP	Leaf, root/NaCl	J.C. Cushman, unpublished
Malate transporter	?	<i>K. daigremontiana</i>	TP	NaCl	Ratajczak <i>et al.</i> , 1994; Steiger <i>et al.</i> , 1997; Lüttge <i>et al.</i> , 2000
Pyrophosphatase	?	<i>M. crystallinum</i>	TP	Leaf, root/-	Mariaux <i>et al.</i> , 1997
		<i>K. daigremontiana</i>			

E. MOLECULES

The final level of scaling considered in this book is represented by the molecules. Addressing molecular ecophysiology of salinity-stress responses, i.e., the molecular basis of eco-physiological reactions, we think of molecules not only as nucleotides as it is often happening in a more narrow view of "molecular biology". Besides nucleotides, which form the genetic basis of halotolerance (Chapters 20, 21, and 22), other molecules such as lipids and proteins are equally important. Compartmentation (see above Chapters 8 and 9) needs membranes. Function of membranes under salinity stress requires modifications of lipids and the proteins of primary active ion pumps (mainly H^+ and Ca^{2+} pumps) and transporters of ions and solutes, such as carriers and channels, as well as their regulation at the transcriptional, translational and post-translational level. The plasma membrane and the tonoplast are the major membranes that function in plant-cell responses to salinity, and they are treated in Chapters 18 and 19. In addition to lipids, proteins and nucleotides other classes of molecules may be important in regulation, e.g. carbohydrates and nitrogen compounds (amino acids, Chapter 11), although to date not much is known about this with respect to salinity. Moreover, many other messenger molecules are involved, such as Ca^{2+} (Chapter 10) and phytohormones (Chapter 13). Thus, molecular ecophysiology requires integrated approaches and much still needs to be learned at the molecular level of salinity-stress responses.

CHAPTER 18

FUNCTION OF MEMBRANE TRANSPORT SYSTEMS UNDER SALINITY: PLASMA MEMBRANE

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Abstract

The plasma membrane is the seat of multiple transport systems which transfer a wide range of solutes into or out of plant cells. These systems mediate both passive and active fluxes; the immediate energy source for the latter is frequently the protonmotive force generated by the plasma membrane H^+ -ATPase. This chapter focusses on the systems which transport K^+ , Na^+ , Cl^- and H_2O (a significant number of which have now been identified at the molecular level), and considers the mechanisms which restrict intracellular Na^+ and Cl^- concentration, and maintain that of K^+ , in the face of high external salinity. Key components of the transduction pathway between the signalling of saline stress and response at plasma membrane level have recently been recognized.

18.1 Introduction

A plant challenged by high ambient salinity will be assisted to survive if it has the ability to perform certain crucial functions, among the most important of which (by common consensus) are:

- to maintain and retain a favorable cytosolic K^+/Na^+ ratio in the face of low K^+/Na^+ ratios in the environment (Chapter 8);
- to prevent Na^+ from reaching harmful (see Serrano *et al.*, 1999) concentrations in the cytoplasm (while at the same time allowing use of the ready availability of Na^+ for osmoregulation of the vacuole; Chapter 8);
- to maintain an adequate net water flux into the cell to support turgor.

It is evident that efficient functioning of the plasma membrane must play a dominant role in meeting these requirements. The plasma membrane is the seat of multiple

transport systems - not only specific systems for the various solutes, organic and inorganic, which are taken up into, or ejected from, the cell, but in some cases multiple systems for a single solute. This arrangement allows dynamic adaptation to a wide range of environmental conditions. The operation of these plasma membrane transport systems results in controlled trafficking of molecules between the cell and its surroundings, and can provide favorable intracellular metabolite concentrations even under severely unfavorable external conditions. Because of its location, the plasma membrane will also play a major role in sensing changes in, and picking up signals from, the environment.

18.2 Passive and active solute fluxes

In order to appreciate the membrane activities involved in the control of internal K^+ , Na^+ and Cl^- concentrations we must identify the forces and mechanisms responsible for the fluxes into and out of cells. Ions move passively under the influence of two forces: the chemical potential gradient ($\Delta\mu$) and the electrical potential gradient ($\Delta\Psi$). The total driving force for passive ion flux across the plasma membrane is thus the electrochemical potential gradient ($\Delta\tilde{\mu}$), which for an ion j is given by:

$$\Delta\tilde{\mu}_j = RT \ln \frac{a_j^o}{a_j^i} + zF\Delta\Psi \quad (1)$$

where a_j^o and a_j^i are the activities of j on the outer and inner sides of the membrane respectively; and z , F , R and T have their usual meanings.

At equilibrium:

$$\Delta\Psi = \frac{RT}{zF} \ln \frac{a_j^i}{a_j^o} \quad (2)$$

This is a useful equation for determining whether or not an ion is passively distributed across the membrane. Passive ion fluxes across the plasma membrane occur largely through ion channels (see Section 18.4.2).

If an ion flux is seen to proceed against the electrochemical potential gradient, it is "active" and dependent on a metabolic energy supply. We are in debt to the prophetic intuitions of Mitchell (1986) for our present day perception of membrane function. We envisage a small number of primary energy transducers, primary ion pumps directly driven by (in most cases) ATP hydrolysis which transfer certain ions across the plasma membrane energetically uphill (primary active flux). Secondary active flux occurs if the return flux of these "working ions" is coupled to the uphill transmembrane flux of other solutes by certain membrane proteins (porters or carriers). The two coupled fluxes may be in the same direction (symport) or in opposed directions (antiport). The principal working ion in animal cells is Na^+ . In plant cells it is the proton. From an evolutionary point of view, this difference probably reflects the fact that typical animal cells evolved

in seawater while typical plant cells evolved in fresh water (Serrano, 1996). The primary ion pump which creates the "protonmotive force" for uptake or ejection of numerous solutes in plant cells is the plasma membrane H^+ -ATPase (see Section 18.3.)

18.3 The plasma membrane H^+ -ATPase

The plasma membrane H^+ -ATPase or proton pump belongs to the class known as P-type ATPases (phospho-ATPases) and its structure and molecular characteristics have been intensively investigated (Serrano, 1989; Sussman, 1994; Palmgren, 1998). Numerous isoforms exist which are expressed to different degrees in different tissues (Sussman, 1994; Palmgren, 1998). The protonmotive force (i.e. $\Delta\tilde{\mu}_{H^+}$) which the pump generates drives the trans-plasma membrane fluxes of a large number of solutes including ions, sugars and amino acids (Reinhold and Kaplan, 1984; Bush, 1993). The proton pump might thus be expected to play a decisive part in re-establishing and maintaining turgor under saline and osmotic stress, and restricting the concentration of toxic ions in the cytosol (Rausch *et al.*, 1996).

While no differences in pump characteristics have been detected between salt-sensitive and salt-tolerant species (Brüggemann and Janiesch, 1987, 1988, 1989), there is a growing number of reports of increased plasma membrane H^+ -ATPase activity in response to external salinity (Braun *et al.*, 1986; Niu *et al.*, 1993 a, b; Binzel, 1995; Ayala *et al.*, 1996; Lin *et al.*, 1997; Wu *et al.*, 1998). Niu *et al.*, (1993 a, b) demonstrated that the increased H^+ -pump activity detected in plasma membrane vesicles from the roots of the halophyte *Atriplex nummularia* after exposure to NaCl (Braun *et al.*, 1986) was associated with higher message accumulation and hence was probably at least partially due to transcriptional regulation. NaCl-induced expression of the plasma membrane H^+ -ATPase was also detectable in the glycophyte *Nicotiana tabacum*, but to a decidedly lesser degree. A possible inference is that the capacity to induce expression of this gene in response to NaCl may be a salt-tolerance determinant.

The pump might also be subject to post translational modulation by phosphorylation/dephosphorylation (see Palmgren, 1998). The C-terminal regulatory domain of the H^+ -ATPase has several potential phosphorylation sites. The purified enzyme is phosphorylated at serine and threonine residues. This fact, together with the report that apparent activation of plasma membrane H^+ -ATPase is blocked by the protein phosphatase inhibitor okadaic acid, brings to mind the recent revelations with regard to calcineurin-like substances (see Section 18.5) and the central importance of phosphorylation/dephosphorylation of proteins in the pathway between saline stress perception and the cell's response to such stress. The important question presents itself - does exposure to saline stress alter the level of phosphorylation of the H^+ -ATPase *in vivo*? Of possible relevance here are the enigmatic 14-3-3 proteins (Palmgren, 1998; Finnie *et al.*, 1999) whose function is not yet understood, but which are ubiquitous in eukaryotes and well conserved throughout evolution. They are thought to activate the plasma membrane H^+ -ATPase after binding to phosphorylated sites.

18.4 Trans-plasma membrane fluxes of K^+ , Na^+ and Cl^-

At the steady state the cytosolic concentration of an ion will largely depend on the balance between the processes effecting entry into the cytoplasmic pool and those effecting exit. (The latter include transfer across the tonoplast into the vacuole as well as metabolic sequestration and in some cases transfer into the xylem for transport to the shoot). Tonoplast transfer systems are dealt with in Chapter 19. In the following sections we shall discuss the various membrane systems believed to be involved in influx and efflux of K^+ , Na^+ and Cl^- and water across the plasma membrane and consider their relationship to saline stress. A general scheme showing the systems is presented in Figure 1. The individual carriers and channels will be related to in detail as they are reached in the sections that follow.

18.4.1 CARRIER MEDIATED TRANS-PLASMA MEMBRANE K^+ TRANSPORT

Carriers are intrinsic membrane proteins which undergo a cycle of conformational changes (see Stein, 1990) for each molecule or ion which they transport (in contrast to channels, which do not change conformation as ions pass along them, see Section 18.4.2.). Carriers include symporters and antiporters (see Section 18.2.) and are thus capable of active transfer of a solute across the membrane.

Cytoplasmic K^+ concentration has often been estimated to be near electrochemical equilibrium with the ambient solution, giving rise to doubts as to the need to postulate any active K^+ uptake process. However, various lines of evidence have indicated that active uptake occurs, notably at external concentrations below 1 mM. Crucial evidence has been provided by experiments in which $\Delta\Psi$, cytoplasmic K^+ concentration, and net inwards K^+ flux were measured concomitantly (Maathuis and Sanders, 1993, 1994; Walker *et al.*, 1996). The active flux may be energized by H^+ -symport (Maathuis and Sanders, 1994).

18.4.1.1 Identification of K^+ transporters

Progress in the identification of K^+ transporters has recently been accelerated by use of molecular approaches, notably identification of plant cDNA clones able to complement yeast strains defective in K^+ uptake. Schachtman and Schroeder (1994) reported the isolation of HKT1 (for high affinity K⁺-transporter) from K^+ -starved wheat roots, a clone showing weak homology to TRK1 and TRK2, (for transporter K⁺), components of the high-affinity plasma membrane K^+ uptake systems in yeast (Ko and Gaber, 1991). A hydropathy plot (which predicts whether a postulated helical protein segment is likely to be in a hydrocarbon milieu or in water) was constructed from the deduced amino acid sequence. The plot predicted about twelve membrane-spanning domains, consistent with the structure of a typical plasma membrane K^+ symporter (shown in Figure 2).

When HKT1 was expressed in yeast or *Xenopus* oocytes it appeared to confer high-affinity Na^+ -coupled K^+ transport (Rubio *et al.*, 1995), the apparent K_m values for K^+

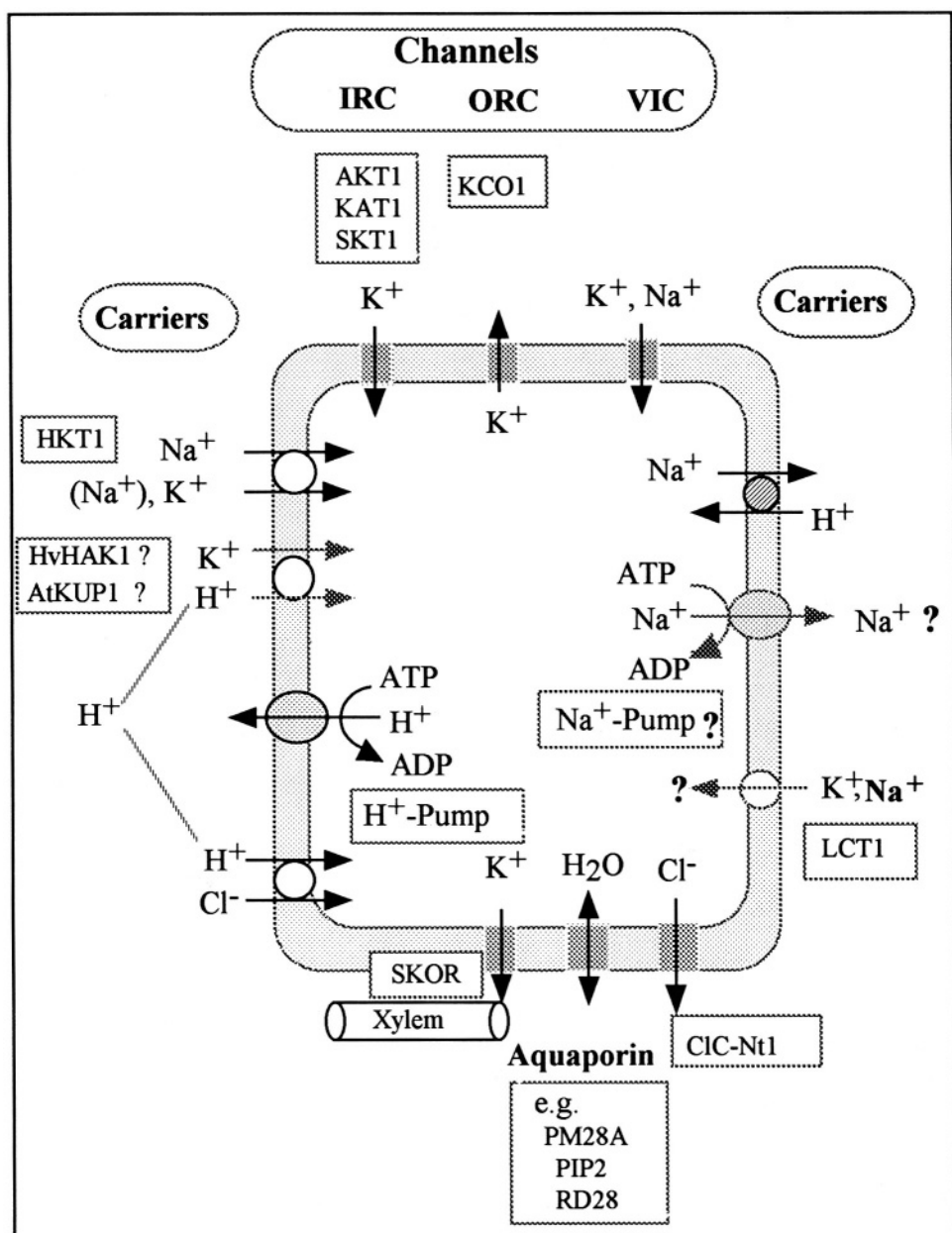


Figure 1. Diagram showing the primary pumps, carriers (symporters and antiporters) and channels postulated to mediate K^+ , Na^+ , Cl^- and H_2O fluxes across the plasma membrane. IRC, inward rectifying channel; ORC, outward rectifying channel; VIC, voltage-independent channel. The mechanisms drawn with lighter lines are less firmly established. For details see text.

and Na^+ being estimated as $3\ \mu\text{M}$ and $175\ \mu\text{M}$ respectively (Rubio *et al.*, 1995; Gassmann *et al.*, 1996). At millimolar extracellular Na^+ concentrations, HKT1 functioned solely as a low-affinity Na^+ transporter due, the authors suggested, to severe competition between Na^+ and K^+ for the K^+ site.

The apparent Na^+ dependence of this K^+ carrier has given rise to spirited debate (Walker *et al.*, 1996; Rubio *et al.*, 1996). It has been pointed out with some vigor that systems coupling Na^+ and K^+ uptake, though recognized in *Chara* and *Nitella* (Smith and Walker, 1989; Walker and Sanders, 1991) have not been detected in any of the terrestrial species examined (Maathuis *et al.*, 1996). (The latter do not seem to have included strongly halophytic species. It was suggested earlier (Reinhold *et al.*, 1985) that it might be rewarding to examine halophytes in this respect).

While the precise physiological importance of HKT1 remains unresolved, further work has added interesting information about structural components involved in NaCl tolerance. Single amino acid changes can increase the K^+/Na^+ selectivity of HKT1, as can be detected after expression in transport-defective yeast cells. Point mutations in the sixth hydrophobic domain decrease low-affinity Na^+ uptake and confer salt tolerance (Rubio *et al.*, 1996). More stringent selection for tolerance was achieved by using a yeast strain deficient in Na^+ extruding ATPases, and therefore highly sensitive to Na^+ (Rubio *et al.*, 1999). Four new HKT1 mutants capable of bestowing a striking degree of salt tolerance showed a mutation at position Asn-365, indicating this site's high importance for determining the interaction of HKT1 with Na^+ , and hence enhanced K^+/Na^+ selectivity. Asn-365 is located in a hydrophilic loop domain (see Figure 2), recalling the findings that in K^+ channels, and in the bacterial K^+ pump Kdp, the loop domains are important for cation selectivity. If HKT1 is indeed found to serve as a significant pathway for Na^+ influx into roots, the information collected in these experiments could be useful for future attempts at genetic engineering for Na^+ tolerance. Meanwhile it is of interest to note the report that expression of an HKT1-type K^+ transporter in rice was depressed during salt stress (Golldack *et al.*, 1997) and was lower in a salt tolerant than in a salt sensitive variety.

A further family of K^+ transporters, the KUP-HAK (for K^+ uptake-high affinity K^+) gene family, has recently come to light in higher plants, identified on the basis of shared sequence homology with known transporters in fungi and bacteria. These transporters show very high K^+ selectivity and may well be of central importance for high affinity K^+ uptake. The AtKUP genes (for *Arabidopsis thaliana* K^+ uptake) were reported almost simultaneously from three laboratories working with *Arabidopsis* (Quintero and Blatt, 1997; Fu and Luan, 1998; Kim *et al.*, 1998), while HvHAK1 (for *Hordeum vulgare* high affinity K^+) was identified in barley (Santa-Maria *et al.*, 1997). The amino acid sequences of these transporters show high homology to the KUP and HAK1 K^+ transporters from the bacterium *Escherichia coli* and the yeast *Schwanniomyces occidentalis*, respectively. Interestingly, the former appears to be a K^+/H^+ symporter (Epstein *et al.*, 1993). AtKUP1 encodes a hydrophobic polypeptide of 712 amino acids, and the hydropathy plot suggests the structure characteristic of a K^+ -transporter, predicting 12 transmembrane spans as in Figure 2. This is consistent with the number predicted for HAK1 and KUP. AtKUP1 was able to complement K^+ transport deficiencies after expression in mutant yeast (Fu and Luan, 1998) and mutant *E. coli* (Kim *et al.*, 1998). Further, transgenic *Arabidopsis* cells overexpressing AtKUP showed

clearly enhanced Rb^+ uptake from K^+ (Rb^+) in the μM range, the data suggesting a K_m of about $22 \mu M$ (Kim *et al.*, 1998). The latter authors concluded that AtKUP probably encodes a high-affinity transporter, though they added that they could not exclude the possibility that the observed high affinity uptake was the result of overexpression of a low affinity transporter. In this conclusion they are mistaken. Overexpression would raise the apparent maximum rate for transport, but it would not be expected to decrease the K_m which would thus still indicate low affinity.

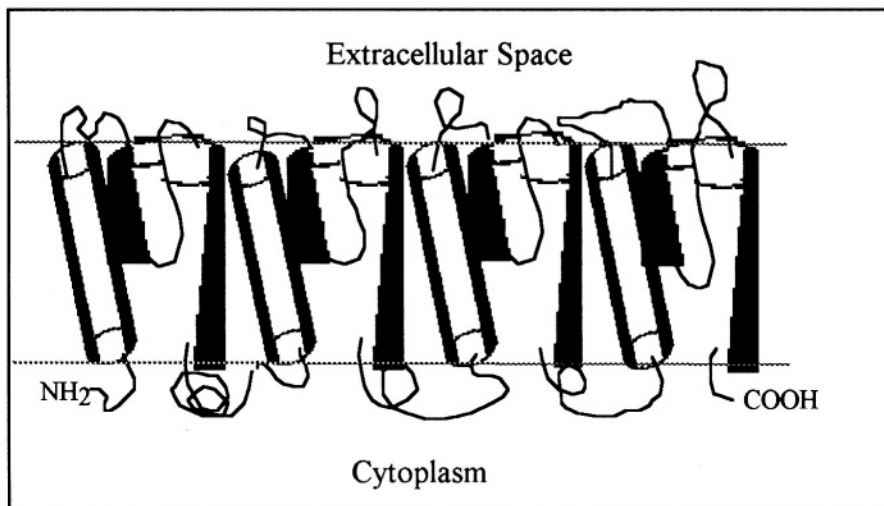


Figure 2. Model of a typical eukaryotic K^+ -symporter showing the 12 membrane-spanning domains (represented by cylinders). The example shown is HKT1 from wheat. The position Asn-365, implicated in the determination of cation selectivity and salt tolerance (see text), is located in the hydrophilic loop between the 8th and 9th transmembrane domains. (Adapted from Durell *et al.*, 1999).

Kinetic studies conducted over a much wider concentration range (Fu and Luan, 1998) led to the striking conclusion that uptake via AtKUP1 was "biphasic". The K_m for the high-affinity phase was $44 \mu M$ and for the low affinity phase $11 mM$. Both phases showed sensitivity to Ba^{2+} , Cs^+ , and tetraethyl ammonium (TEA). Though it is possible that these inhibitors might affect other transporters as well (Hille, 1992) they are usually regarded as K^+ channel blockers (voltage dependent channels in the case of TEA). The possibility therefore arises that AtKUP1, though clearly conforming to the K^+ symporter family structure, might also carry the structural elements of a functional K^+ channel (see Section 18.4.2). This recalls Nissen's proposal (in line with current concepts regarding multistate ion channels, Lauger, 1984) that solutes are transported across the plasma membrane by entities with multiple conformational states, having carrier-like properties at low external solute concentrations and channel-like properties at high concentrations (Nissen, 1989, 1991).

There has been a marked tendency to identify the various K^+ transport systems now being uncovered with one or other of the two phases of Epstein *et al.*'s (1963) classic curves for uptake versus concentration, which were interpreted as indicating the

presence of two discrete systems working in parallel. However, caution is called for. The single protein studied by Fu and Luan (1998) appeared to show biphasic kinetics as discussed above, and complex kinetics were also reported for the single protein AKT1 (Sentenac *et al.*, 1992 see Section 18.4.2). The kinetics for transport of many solutes are non-Michaelian, and a number of interpretations have been considered (see Reinhold and Kaplan, 1984). It would be interesting to examine the kinetics for HKT1 observed over a wider concentration range.

Both in the "high-affinity" and the "low-affinity" K^+ -concentration range, K^+ uptake mediated by AtKUP1 was strongly inhibited by NaCl concentrations of 5 mM and higher. Fifty mM of NaCl brought about 90% inhibition of "high-affinity" uptake. The NaCl concentrations effective in the case of AtKUP1 are similar to those which inhibit uptake by plant roots (Epstein, 1973; Wu *et al.*, 1996) a fact which encourages the authors to propose that AtKUP1 functions as a major K^+ transporter *in vivo* (Fu and Luan, 1998). Inhibition by NaCl was also reported for HvHAK1 (Santa-Maria *et al.*, 1997). The authors infer that this inhibition was "competitive", but they present no kinetic analysis to validate this inference. HvHAK1 was shown to be capable of transporting Na^+ when the latter was present in the millimolar range.

18.4.2 PASSIVE K^+ FLUX THROUGH CHANNELS

Channels are intrinsic membrane proteins which function as selective pores. Unlike carriers (see Section 18.4.1) they do not undergo conformational changes during the transmembrane solute fluxes which they mediate (see Stein, 1990). Maximal velocity of transport via a channel is as a consequence orders of magnitude higher than that achieved by carriers (10^6 to 10^8 ion s^{-1}). It is now emerging that a number of sequences representative of K^+ channels show extensive homologies with carrier-type active transport systems, including the TRK system in yeast and HKT1 in wheat (Durell *et al.*, 1998; Durell and Guy, 1999). It is likely that several important classes of K^+ carriers are structural variants of well defined K^+ channels. This may have relevance to the observation that K^+ channel blockers inhibit transport mediated by the carrier AtKUP1 (see Section 18.4.1.1).

Much of our present understanding of the characteristics and behavior of channels has been gained by application of the "patch clamp technique" for observing transmembrane ion currents. (For a helpful discussion of this methodology see Tyerman and Skerrett, 1999). Flux through a channel can be regulated by means of a "gating" or closing process which is frequently dependent on voltage. Thus the proportion of time that a plasma membrane voltage-controlled channel is open is a function of the plasma membrane potential. Gating is also dependent on the concentration of the permeating ion, and these two dependencies results in the phenomenon known as "rectification" i.e. a channel will appear to allow ion flux only in the inward or outward direction (inward rectifier or outward rectifier respectively).

18.4.2.1 Inward rectifying channels

The inward rectifying channels in the plasma membrane appear to function principally in K^+ uptake (Findlay *et al.*, 1994 ; Maathuis *et al.*, 1997). These channels are open when the plasma membrane potential becomes more negative than the equilibrium

potential for K^+ , allowing the passive influx of K^+ down its electrochemical potential gradient. The first plant K^+ -transporting proteins characterized at the molecular level turned out to be inward rectifying channels. AKT1 (Sentenac *et al.*, 1992) and KAT1 (Anderson *et al.*, 1992) were cloned from *Arabidopsis* by functional complementation of yeast strains defective in K^+ transport. AKT1 is preferentially expressed in the plasma membrane of peripheral root cells (Lagarde *et al.*, 1996) and presumably has a role in K^+ uptake into the plant. KAT1 is expressed in leaf guard cells (Nakamura *et al.*, 1995) and is likely to play a central part in stomatal movement. Down-regulation of K_{in} channels is associated with stomatal closure, one of the immediate responses to salinity, reducing water loss through transpiration and diminishing ion flux to the shoot.

Both AKT1 and KAT1 share the structural design of the *Drosophila* "Shaker" channel, and both show very high specificity for K^+ over Na^+ ($P_{Na^+}/P_{K^+} = 0.05$; Véry *et al.*, 1995, Bertl *et al.*, 1997). Interestingly, an *Arabidopsis* AKT1 null mutant showed greater deficiency in K^+ (Rb^+) uptake at micromolar than at millimolar external concentrations (Hirsch *et al.*, 1998) demonstrating high affinity uptake by the channel, and contradicting the frequently accepted view which identifies channel-mediated uptake with low-affinity uptake. Even at micromolar external K^+ concentrations, a driving force for passive inward K^+ flux existed, since the membrane potential was observed to be extremely negative (-250 mV, Hirsch *et al.*, 1998).

A K^+ channel, KST1 (for K^+ *Solanum* transporter), has also been cloned from potato leaf epidermal tissues enriched in guard cells (Müller-Röber *et al.*, 1995). It shares many properties with KAT1 and its electrical properties, when expressed in *Xenopus* oocytes, are virtually identical to those of the major inwardly-rectifying K^+ channels of potato guard cells.

Bertl *et al.* (1997) observed in studies of AKT1 expressed in yeast that brief replacement of extracellular K^+ by Na^+ enhanced the subsequent K^+ currents by nearly 100%. This suggests that ions may bind to modulator sites on the channel itself, and persist there after removal from the medium, regulating gating. It also suggests the possibility that the capacity for K^+ uptake might be raised in response to salinity. In view of the high level of expression of AKT1 in roots, these findings may have significance for resistance to saline stress. K^+ uptake has been implicated in salt tolerance in studies at the level of cell lines in suspension culture (Watad *et al.*, 1986; 1991) and at that of cultured gametophytes of a single gene mutant of the fern *Ceratopteris richardii* (Warne *et al.*, 1996).

18.4.2.2 Outward rectifying channels

In contrast to inward rectifying channels, outward rectifying channels open when the membrane potential becomes more positive than the equilibrium potential for K^+ . K^+ will therefore pass out of the cytoplasm along its altered electrochemical gradient. The gradient is inwards in the case of Na^+ (see next Section) and net influx of Na^+ may consequently occur through these channels if they are open as result of depolarization of the membrane under saline conditions (Cakirlar and Bowling, 1981). The relative specificity of the channel for K^+ and Na^+ respectively might thus plausibly be a factor in salt tolerance. However, wheat genotypes differing in their salt tolerance failed to show differences in the K^+/Na^+ selectivity of the outward rectifier (Schachtman *et al.*, 1991), and this was also true for salt-adapted and wild type cell lines (Murata *et al.*, 1994;

Amtmann *et al.*, 1997). On the other hand Murata *et al.* (1994) reported a decrease in depolarization-activated outward K^+ current in NaCl-adapted tobacco cell lines. They inferred a reduction both in K^+ leakage and in Na^+ entry in the NaCl adapted line, mitigating salinity damage.

Two plant outward rectifiers have been identified at molecular level. One, designated KCO1 (for K^+ channel out, Czempinski *et al.*, 1997), was expressed in insect cells and observed to be activated by concentrations of free cytosolic Ca^{2+} in the physiological range. Opening of this channel would be expected to be one consequence of the rise in free cytosolic Ca^{2+} concentration elicited by salt stress (Lynch *et al.*, 1989). A second plant outward rectifier, SKOR (for stelar K^+ outward rectifier, Gaymard *et al.*, 1998) is highly expressed in the root pericycle and is believed to be involved in K^+ transfer into the xylem for translocation to the shoot. Transgenic *Arabidopsis* plants with knockout mutations with SKOR show decreased shoot K content. Absciscic acid (ABA), a hormone associated with response to saline and water stress, reduces SKOR expression levels.

18.4.3 INFLUX AND EFFLUX OF SODIUM

When external salinity is moderate to high (>100 mM Na^+) the cytoplasmic Na^+ concentration is usually well below the predicted value for electrochemical equilibrium of this ion across the plasma membrane. Thus, in striking contrast to the case of K^+ (and also Cl^- see below) a strong driving force exists in the cell at steady state for passive inward flux of Na^+ . Efflux, on the other hand, must occur energetically uphill against the electrochemical potential gradient (see e. g. Schubert and Läuchli, 1990, who produced evidence for active Na^+ efflux by comparing flux ratios in maize root with values predicted by the Ussing-Teorell equation for passive flux). Efflux must therefore be mediated by a plasma membrane mechanism dependent on metabolic energy supply. The evidence so far to hand suggests that efflux is secondary active transport, as is the case in many bacteria. Protons that have been extruded across the plasma membrane by the primary proton pump re-enter the cell down their electrochemical gradient, driving Na^+ ions outward by a coupled Na^+/H^+ exchange. Data suggesting the existing of antiport in plant cells emerged from studies of Na^+ fluxes in whole cells or tissues as a function of external pH (Colombo *et al.*, 1979; Jacobi and Teomi, 1988; Mermen *et al.*, 1990) or of H^+ fluxes in cultured cells in response to cation supply (Watad *et al.*, 1986). More rigorous direct evidence for antiport was obtained for root plasma membrane vesicles isolated from the halophyte *Atriplex nummularia*, and from a relatively Na^+ tolerant glycophyte, *Pisum* (Braun *et al.*, 1988; Hassidim *et al.*, 1990). After a ΔpH had been established across the vesicle membrane, addition of Na^+ brought about the dissipation of ΔpH but not of $\Delta \Psi$. It was checked that the indicated Na^+/H^+ exchange was not due to nonspecific electric coupling, nor to competition for anionic adsorption sites on the membrane, nor to inhibition of the H^+ -ATPase; it was therefore concluded that it was antiporter-mediated. Confirmatory evidence for Na^+/H^+ antiport in root plasma membrane vesicles has been obtained by Allen *et al.* (1995) for wheat roots and Wilson and Shannon (1995), for a glycophytic and a halophytic species of tomato, when grown under saline conditions. Isolation of the antiporter has not so far been achieved, but may well follow swiftly after the report (Shi *et al.* 2000) that the gene SOS1 (see section 18.5) encodes a putative plasma membrane Na^+/H^+ antiporter.

The possibility that primary extrusion pumps may also exist in plant cells cannot be dismissed. Electrogenic Na^+ efflux has been inferred from experiments on corn roots (Cheeseman, 1982). Further, a Na^+ -activated ATPase has been identified in plasma membrane preparations from the marine alga *Heterosigma akashiwo* (Wada *et al.*, 1989) and has been shown to cross react with antiserum against animal Na^+/K^+ -ATPase. While no biochemical evidence for Na^+ -ATPase has yet been obtained for higher plants, Serrano (1996) has made the interesting suggestion that the products of the putative Ca^{2+} -ATPase genes identified in tomato (Wimmers *et al.*, 1992) and tobacco (Perez-Prat *et al.*, 1992), which are induced by NaCl, might be involved in Na^+ efflux and should be investigated in a suitable expression system. This suggestion is based on the observation that the yeast gene ENA12/PMR2 (for efflux Na^+) which shows homology to genes encoding animal ATPases, is also induced by NaCl and that its product participates in Na^+ efflux.

Keeping the intracellular Na^+ concentration low may require not only efficient mechanism(s) for extruding Na^+ from the cells but a low rate of Na^+ entry. The route for Na^+ influx, clearly passive down its electrochemical gradient, has not yet been unambiguously identified in plant cells. No Na^+ -specific channel has been detected by patch clamp techniques, and the dominant cation channels in general show high K^+/Na^+ specificity, though the K^+ outward rectifier is in some plants less selective for K^+ and can open to allow inward K^+ or Na^+ currents under some circumstances. Attention has recently been focusing on voltage-independent cation channels in root plasma membranes (Tyerman and Skerrett, 1999; Amtmann and Sanders, 1999). These only occur in small numbers but are relatively non-selective. In an interesting calculation which integrates information on the conductance, selectivity and abundance of various channel types, Amtmann and Sanders (1999) have demonstrated that voltage-independent channels make the major contribution to Na^+ current under high salt conditions. In fact these channels might have to be down-regulated if excessive Na^+ uptake under salinity is to be avoided (White and Ridout, 1995).

The possibility has been explored that neurotoxins which bind specifically to animal Na^+ channels might assist in the detection of possible plant homologues (Friedman and Reinhold, 1995). The rationale for this approach was the observation that the cloned plant K^+ channels, e. g. AKT1 and KAT1, are homologous to nerve cell K^+ channels, and voltage gated Na^+ channels are similarly highly conserved in the animal kingdom. In the facultative halophyte *Mesembryanthemum crystallinum*, where NaCl is the trigger for switches in metabolic pathways, plasma membrane Na^+ channel frequency might be high. Plasma membrane vesicles isolated from *Mesembryanthemum* roots were therefore chosen as experimental material. (Most of the patch clamp studies have been conducted on glycophytes). The results indicated specific binding of the alkaloid batrachotoxinin-A 20- α -benzoate, which binds to site 2 in animal Na^+ channels.

Evidence has also been produced for carrier-mediated Na^+ uptake. Low-affinity Na^+ influx mediated by the carrier-type transporter HKT1 during salt stress has already been referred to in Section 18.4.1.1. Additional cation transporters which may contribute to low affinity Na^+ influx are HvHAK1 from barley (Santa-Maria *et al.*, 1997, see Section 18.4.1.1.) and LCT1 (for low-affinity cation transporter, Schachtman *et al.*, 1997). The latter, isolated from a wheat root cDNA library, appears to be relatively non-selective for cations and also transports divalent cations such as calcium.

18.4.4 CHLORIDE TRANSPORT

Largely owing to the negative plasma membrane potential, the electrochemical potential gradient for Cl^- is uphill into the cell under non-saline conditions. Uptake can only be achieved by means of an active mechanism, thought to be Cl^-/H^+ symport with a stoichiometry of more than one H^+ per Cl^- (Sanders, 1980; Felle, 1994). The acquired Cl^- is necessary for nutrition, as well as for osmotic adjustment of the vacuole and for charge balance. However, a significant rise in cytosolic Cl^- concentration such as could result from exposure to salinity would be harmful to cellular systems (see Serrano *et al.*, 1999) and it seems probable that mechanisms exist to bring about Cl^- efflux from the cell. Various anion channels have been identified by patch clamp techniques (see Tyerman and Skerrett, 1999) regulated by voltage or calcium, which might open to allow efflux driven by the downhill outward electrochemical potential gradient. A Cl^- channel has also been characterized at the molecular level. ClC-Ntl (for Cl^- channel *Nicotiana tabacum*), a plant homologue of animal voltage-dependent chloride channels, was cloned by PCR strategy (Lurin *et al.*, 1996). When expressed in *Xenopus* oocytes it activated slowly upon membrane hyperpolarization.

There is indirect evidence that anion channels do indeed open to allow Cl^- efflux under salinity. Salt stress-induced enhancement of Cl^- permeability was noted by Yamashita *et al.* (1994). Boursier and Lauchli (1989) have reported the extrusion of Cl^- from the roots of sorghum plants. At very high salinities it has been estimated that the electrochemical potential gradient for Cl^- might possibly be reversed, allowing passive Cl^- influx into the cells (e. g. Binzel *et al.*, 1988; Skerrett and Tyerman, 1994). We shall need more accurate measurements of the changes in cytosolic Cl^- concentrations with time after exposure to saline stress in order to unravel the way in which Cl^- concentration is regulated, and to clarify the roles of the various putative Cl^- transport mechanisms in salt tolerance.

18.5 The modulation of K^+ and Na^+ transport in adaptation to saline stress

Until recently very little was known about the signalling system that allows a plant to sense rising external salinity; or about the response mechanism that brings about appropriate modulation of the transmembrane ion fluxes. But the last few years have seen a great leap forward. The principal players in the unfolding story appear to be calcium- and calmodulin- dependent protein phosphatases, resembling calcineurin in animals and fungi. Calcineurin, a serine-threonine protein phosphatase, is a heterodimer made up of a regulatory B subunit (CNB) and a catalytic A subunit (CNA), and is a major regulator of various cellular processes. It is activated when it binds Ca^{2+} and calmodulin (Klee *et al.*, 1988) and is a well-characterized effector of calcium signalling in both animals and fungi.

Important studies on *Saccharomyces cerevisiae* have established the central role played by calcineurin in salt tolerance in yeast. The general level of expression of the gene *ENA1*, encoding a plasma membrane-located Na^+ -ATPase primarily responsible for pumping Na^+ out of the cell, is specifically dependent on Ca^{2+} /calmodulin-activated calcineurin (Mendoza *et al.*, 1994; Serrano, 1996, Serrano *et al.*, 1999). Further,

activated calcineurin modulates the plasma membrane TRK system responsible for most of the high-affinity K^+ uptake in *S. cerevisiae*. As a result the affinity of the system for K^+ is increased but not that for Na^+ or Li^+ (see Serrano, 1996). K^+/Na^+ discrimination is thus sharply increased. This calcineurin-mediated enhancement of Na^+ influx, and depression of Na^+ uptake, together lead to restricted Na^+ accumulation in spite of high external Na^+ concentration.

Evidence that the development of salt tolerance in plants follows a similar scenario is now accumulating with impressive speed. Immunosuppressive drugs which target serine/threonine protein phosphatases have been used by researchers as probes for the functioning of such proteases, and the results have provided evidence that calcineurin-like substances regulate plasma membrane K^+ channels in guard cells and other leaf cells (Luan *et al.*, 1993; Li *et al.*, 1994). Moreover, supply of calcineurin of animal origin has been shown to modulate ion flux through the plasma membrane in guard cells (Luan *et al.*, 1993). Two *Arabidopsis* genes have been identified, STO and STZ (for salt tolerance and salt tolerance zinc finger respectively), which confer increased tolerance to LiCl and NaCl on yeast calcineurin mutants which are salt sensitive (Lippuner *et al.*, 1996). Pardo *et al.* (1998) have taken an opposite experimental approach - they have succeeded in reconstituting activated yeast calcineurin in transgenic tobacco plants by co-expressing a truncated form of the yeast catalytic subunit and the regulatory subunit in the higher plants. The transgenic tobacco plants were strikingly more NaCl tolerant (see Figure 3), leading the authors to conclude that the activated yeast calcineurin functioned in apparent conjunction with a salt-stress signalling pathway present in plants, and as a result mediated salt tolerance.

Studies of salt-hypersensitive mutants of *Arabidopsis* (Wu *et al.*, 1996; Liu and Zhu, 1997; Zhu *et al.*, 1998) have brought about further progress. These interesting mutants, SOS1, SOS2, and SOS3 (for salt overly sensitive), combine an inability to grow under low K^+ conditions with hypersensitivity to NaCl, and there is evidence suggesting impaired high-affinity K^+ transport ability. The SOS3 mutant also appeared to show a decrease in K^+/Na^+ selectivity. (The figures presented, however, are flawed in that the possible apoplastic location of part of the Na^+ detected has not been taken into account. Brief rinsing in water will not remove ions adsorbed to sites in the Donnan Free Space of the wall, and after treatment in 50 mM NaCl such adsorption may make a substantial contribution to the total Na^+ detected). An increase in Ca^{2+} in the medium not only restored the ability of SOS3 plants to grow at low K^+ concentrations but partially suppressed the Na^+ -hypersensitivity. The sequence of the SOS3 gene (Liu and Zhu, 1998) indicates a gene product sharing highest sequence homology with the B subunit of calcineurin and animal neuronal Ca^{2+} sensors. Recent rapid developments have revealed that the SOS2 gene encodes a serine/threonine protein kinase (Liu *et al.* 2000). SOS3 binds to this kinase and activates it in the presence of Ca^{2+} (Halfter *et al.* 2000). The system thus stands in contrast to calcineurin, where the regulatory subunit CNA binds to and activates, not a kinase, but a protein phosphatase. It has also been reported (Shi *et al.* 2000) that SOS1 encodes a transmembrane protein with similarities to plasma membrane Na^+/H^+ antiporters from bacteria and fungi. The expression of SOS1 under NaCl stress appears to be controlled by the SOS3/SOS2 regulatory pathway.

Kudla *et al.* (1999) have attempted to identify plant calcineurin by a systematic PCR approach, concentrating on the CNB subunit since the latter is highly conserved, and

contains unique sequences absent from other Ca^{2+} binding proteins. AtCBL1 (*Arabidopsis thailana* calcineurin B-like protein 1) was cloned from an *Arabidopsis* cDNA library and shows highest similarity with CNB from animals. Protein-protein interaction studies indicate that it is able to react with the CNA unit from rats. Very interestingly, rat CNA combined with AtCBL1 restored salt tolerance to yeast CNB mutants. Northern blot analysis showed that expression of AtCBL1 was strongly regulated by various stress signals and was highest in roots and stems. AtCBL1 and SOS are not identical, though apparently closely related. No plant homologue of the calcineurin catalytic subunit A has yet been identified (Serrano *et al.*, 1999).

In sum, the accumulating evidence strongly suggests a critical role for compounds resembling calcineurin in the pathway between salinity perception and the resulting modulation of plasma membrane ion transport systems. It has been recognized for some time that the selectivity of K^+/Na^+ uptake under saline conditions is Ca^{2+} dependent (LaHaye and Epstein, 1969), and that salt stress elicits a sudden rise in cytosolic Ca^{2+} concentration (Lynch *et al.*, 1989). The mechanism by means of which Ca^{2+} fulfills its role as a second messenger in this system is becoming clearer with the discovery of the Ca^{2+} -dependent plant calcineurins (see Bressan *et al.*, 1998).

18.6 Control of water flux across the plasma membrane

A saline environment presents the challenge of osmotic stress in addition to potentially harmful Na^+ and Cl^- concentrations. Successful accommodation involves regulating water flux into the cells so as to maintain turgor and support growth in the face of such stress. It is only very recently that we have begun to understand how water flux may be controlled at the level of membranes. Water moves across the plasma membrane passively, in response to the gradient in water potential, the two principal components of which are the hydrostatic potential and the osmotic potential. Transmembrane water flux (J_v) is given by the equation:

$$J_v = L_p (\Delta P - \sigma \Delta \pi) \quad (3)$$

where L_p is the coefficient of hydraulic conductivity (proportional to the osmotic permeability), ΔP and $\Delta \pi$ are the gradients in hydrostatic and osmotic potential respectively, and σ is the reflection coefficient (see Dainty, 1963).

Until recently it was envisaged that water crossed biological membranes without the aid of specific channels or transporters. However, it had been noted that there was a discrepancy between the observed permeability of the membranes to water moving down an osmotic (or hydrostatic) transmembrane gradient (osmotic permeability), and that measured for water exchange in the absence of a gradient (diffusional permeability); and this had been interpreted as evidence for the existence of water-filled pores (see Ray,

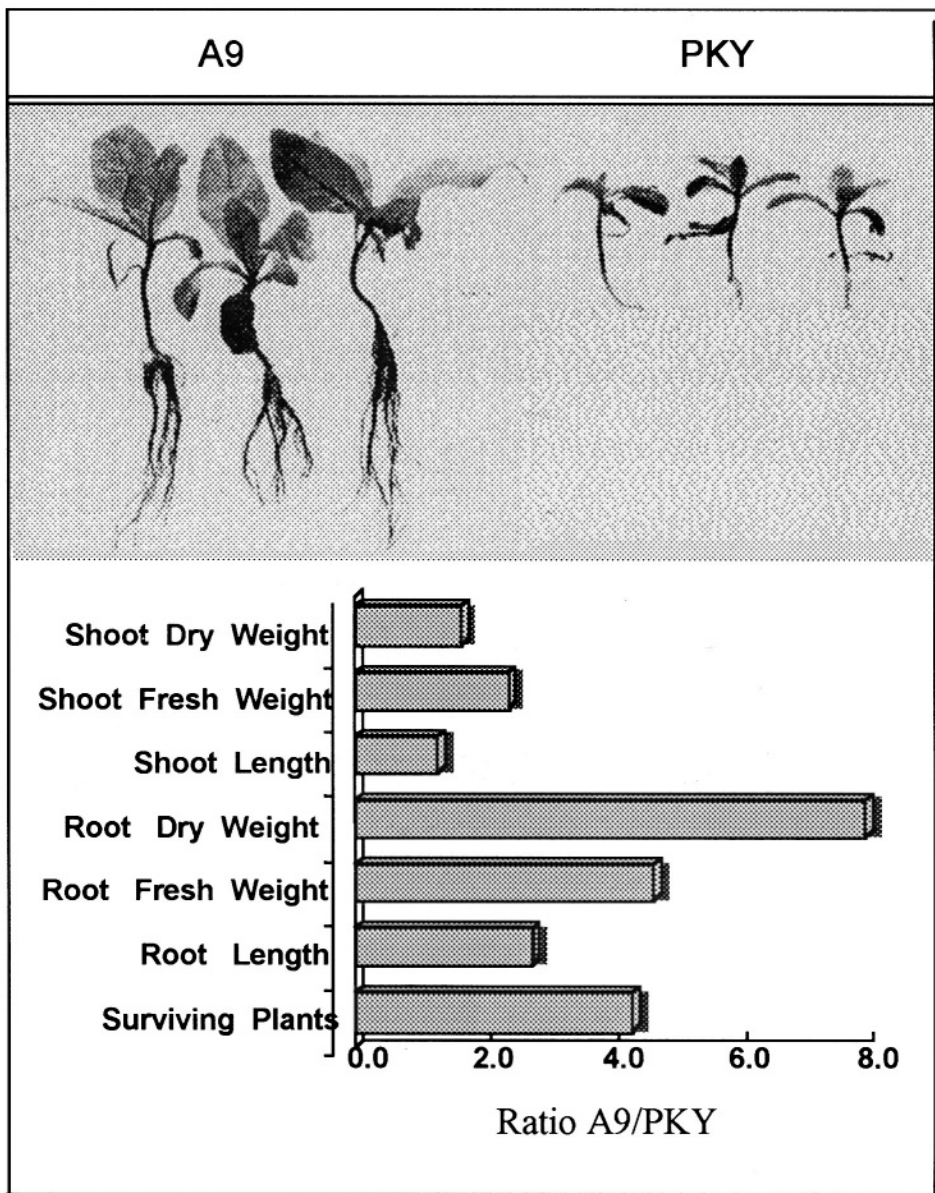


Figure 3. Enhanced salt tolerance shown by transgenic tobacco plants co-expressing truncated form of the calcineurin catalytic subunit from yeast and the regulatory subunit. **Above**, representative plants from line A9 (expressing the yeast calcineurin genes as well as neomycin phosphotransferase as a selectable marker) and PKY (expressing only the selectable marker) photographed after 7 days of treatment with 200 mM NaCl, followed by 12 days of recovery. **Below**, comparative survival and growth parameters for the two sets of plants. Initially 36 plants in each set. (Adapted from Pardo *et al.*, 1998).

1960). The discovery of water channels was also clearly foreshadowed in the deductions made by Wayne and Tazawa (1990) from their experiments on the Characeae.

The breakthrough came in 1992 with the discovery of water channel proteins (aquaporins), i. e. intrinsic membrane proteins which specifically facilitate transmembrane water flux, in the erythrocyte plasma membrane (Preston *et al.*, 1992). They have since been documented in plants, animals and bacteria. The aquaporins belong to the Major Intrinsic Protein (MIP) family. Plasma membrane intrinsic proteins (PIPs) form a subgroup of the family, distinguished on the basis of amino acid sequence. It has been demonstrated that about 20% of the integral proteins of spinach leaf plasma membrane are PIPs (Johansson *et al.*, 1996). Studies of three dimensional structure indicate that all MIPs contain six trans-membrane domains with both C and N-terminals at the cytoplasmic surface of the membrane (see Figure 4). MIPs are also characterized by the signature sequence Asn-Pro-Ala which occurs in the loop between the second and third membrane-spanning domain, and again in the loop between the fifth and the sixth. It is believed that these two loops play a part in forming the pore through which the water flux occurs. The proteins form tetramers in the membrane, but each monomer is thought to mediate flux.

In general, trans-plasma membrane water flux would not be expected to rate limit accommodation to osmotic (or saline) stress at cell level. The transmembrane flux of ions and other osmolytes is considerably slower than water flux, as are metabolic changes resulting in altered internal osmotic potential. However, in whole plant or plant tissues, especially where water flow by way of a trans-cellular route dominates the overall water flow, flux through the plasma membrane might well be a limiting factor. The reported control of aquaporin gene expression by salinity or water stress (see below) constitutes a persuasive argument for a role for these plasma membrane transport proteins in turgor maintenance under stress conditions. The same is true for reported translational control by stress. Moreover, the expression pattern for some aquaporins accords with identified sites where water flow might be anatomically constrained. For instance, in the root their abundance is high in xylem parenchyma cells, endodermis and outer cortical layers (Kaldenhoff *et al.*, 1995; Yamada *et al.*, 1995).

18.6.1 TRANSCRIPTIONAL AND POST-TRANSLATIONAL REGULATION BY WATER OR SALINITY STRESS

The evidence, so far, for transcriptional control is somewhat fragmentary. Saline stress down-regulated aquaporin expression in *Mesembryanthemum crystallinum* (Yamada *et al.*, 1995). Both up-regulation by water stress (Guerrero *et al.*, 1990; Yamaguchi-Shinozaki *et al.*, 1992) and down-regulation (Yamada *et al.*, 1995) have been reported, while one plasma membrane aquaporin, PIP1b (Kaldenhoff *et al.*, 1993, 1995) is induced by ABA, a hormone associated with saline and water stress.

Evidence has also been brought for the attractive possibility that stress exerts post-translational regulation of aquaporins via phosphorylation-dephosphorylation of these membrane proteins - attractive because it falls into line with present day views on the role of protein phosphatases in the pathway between saline or water stress perception and the modulation of plasma membrane ion transport (see Section 18.5.). Johansson *et al.* (1996, 1998) have reported that one of the most abundant proteins of the spinach leaf

plasma membrane (designated PM28A and sharing considerable homology with the plasma membrane aquaporins RD28 and PIP2) is an aquaporin, and that its water channel activity is regulated by phosphorylation at two different sites: ser-115, a serine residue conserved in all plant plasma membrane aquaporins, and ser-274, conserved in the PIP2 subfamily. Importantly, they observed that the level of phosphorylation *in vivo* changed in response to osmotic stress, ser-274 apparently being the site concerned. The authors have outlined a model for the role of PM28A in controlling cellular water balance (Johansson *et al.*, 1998). When the osmotic concentration in the apoplast is low, a stretch-activated Ca^{2+} channel in the plasma membrane (acting as an osmosensor) admits Ca^{2+} which activates the membrane-associated protein kinase which phosphorylates PM28A. The channel is then open, and water flux across the plasma membrane is virtually unrestricted. Under osmotic stress, however, cell turgor falls, the Ca^{2+} channel is closed, the protein kinase thereby inactivated, the aquaporin is dephosphorylated and water flow through the aquaporin is restricted. This, the authors suggest, would hinder water loss and "buy time" for osmotic adjustment. (They also take into account that osmosensing and subsequent signal transduction might be more complex perspectives.)

We now have persuasive evidence that substances resembling calcineurin, a crucial intermediate in a salt-stress signalling pathway in yeast, and an essential component in various Ca^{2+} -dependent signal transduction pathways in animals, are also present in plants. One of them has been shown to be critically involved in K^+ transport and in salt tolerance. A plant Ca^{2+} sensor, capable of picking up and transducing the Ca^{2+} signal triggered by saline stress, has thus been identified. Further work will elucidate the extent to which CNA-like protein phosphatases, as distinct from SOS2 type protein kinases, participate in the mediation of stress responses in plants.

Turgor and Na^+ sensing are clearly central to ion homeostasis under saline stress. Turgor sensors have been recognized in yeast (Hohmann, 1997). They are membrane proteins (a histidine kinase and an SH3 protein), and similar membrane proteins probably exist in plants. It was suggested some years ago (Reinhold *et al.*, 1984), on the basis of the observed sensitivity of the plasma membrane H^+ -ATPase to turgor, that this proton pump could function as detector of turgor change, and, moreover, as effector of osmoregulation via the energization of solute fluxes. This dual function of the H^+ -ATPase would obviate the postulated need (Bisson and Gutknecht, 1980) for information transfer between detector and effector in osmoregulation.

We do not yet know of a Na^+ sensor in plant cells, nor has one been unambiguously identified in yeast. In *E. coli*, the regulator of the NhaA Na^+/H^+ antiporter acts both as sensor and transducer of the Na^+ signal (Carmel *et al.*, 1997).

Modulation of plant membrane K^+ transport systems could be effected at transcriptional or post-translational level. Accumulated evidence for guard cells suggests that here post-translational control dominates, and may well be exerted by altering the phosphorylation state of plasma membrane K^+ channels. We do not have clear-cut information for root cells. The observed increase in plasma membrane H^+ -ATPase activity in response to saline stress might also be due in part to post-translational phosphorylation/dephosphorylation - we await experiments to investigate this possibility. Another open question is the role of 14-3-3 proteins, often found in

association with the H^+ -ATPase in plasma membranes, and known to take part in its regulation after binding to phosphorylated motifs (Finnie et al 1999). The 14-3-3 proteins are thought to function in response to stress signals including saline stress.

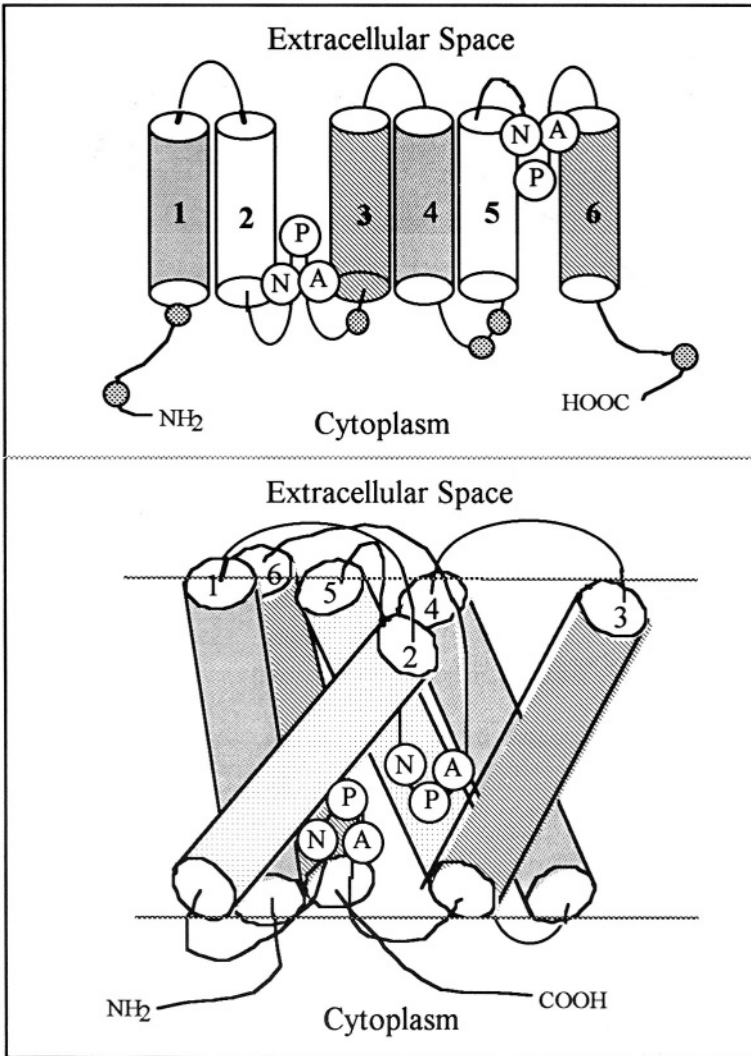


Figure 4. Above, Model of aquaporin monomer (PM28A). The cylinders represent 6 membrane-spanning α -helices. The shading of the helices reflects the internal homology. The filled circles represent serine residues at potential phosphorylation sites involved in the regulation of H_2O flux. The two highly conserved Asn-Pro-Ala (NPA) motifs are indicated. (Adapted from Johansson *et al.*, 1998 and Tyerman *et al.*, 1998). **Below,** topology of aquaporin in the membrane. The shading again reflects the internal homology of the helices. The model is based on the structure for AQP1 (adapted from Kjellbom *et al.*, 1999)

The phosphorylation state of aquaporins has also been reported to be altered as a response to osmotic stress. Might a change in phosphorylation state be the basis of the observed effect of the plant hormone ABA on hydraulic conductivity of plant tissues to water (Glinka and Reinhold, 1971)? It is known that a protein phosphatase (phosphatase 2C) is involved in ABA modulation of K^+ channels. The increase in coefficient of hydraulic conductivity brought about by ABA might also be connected with its reported induction of the plasma membrane aquaporin PIP16 (Kaldenhoff *et al.*, 1993, 1995).

Transcriptional control of membrane transport systems by salt stress is highly likely. Marked rises in the mRNA levels for HvHAK1, AtCUP3 and HKT1 have been reported in roots after K^+ withdrawal (Santa-Maria, 1997; Kim *et al.*, 1998; Golldack *et al.*, 1997). This suggests that the allied stress of high salinity may provoke the same response.

The long-awaited identification of the tonoplast Na^+/H^+ antiporter at molecular level may now have been achieved (Apse *et al.*, 1999), and the finding that SOS1 encodes a putative plasma membrane Na^+/H^+ antiporter may soon lead to the identification and cloning of its plasma membrane counterpart. This achievement would allow new approaches towards determination of the antiporter's role in ejection of Na^+ from the cell. Serrano *et al.*, (1999) have speculated that when Na^+ extrusion occurs via Na^+/H^+ antiport, H^+ -ATPase activity, which generates the driving force for antiport, is induced by saline stress. When, however, Na^+ extrusion occurs via a primary Na^+ extrusion pump, the H^+ -ATPase is of little relevance and its activity will not be increased. A primary Na^+ pump has still proved elusive in plant cells. Following up Serrano's further suggestion that putative plant Ca^{2+} -ATPases induced by NaCl may in fact pump Na^+ (see Section 18.4.3) might bring one to light.

With regard to the enigmatic route of Na^+ entry into cells, we need to determine whether, in halophytes at least, Na^+ specific channels are present. Plant homologues of animal Na^+ channels might be sought by PCR strategy analogous to that which successfully detected the plant chloride channel CIC-Nt1.

We are only at the beginning of the road towards understanding the mechanisms involved in the signalling of saline stress, the transduction pathway, and the response at plasma membrane level. As is so often the case, researchers into microbial and animal cells are ahead of us on this road. The messages they send back to us are challenging. It is up to us to discover where plant cell mechanisms conform and where they diverge in this area where many fundamental steps and components, especially of the signalling machinery, appear to have been conserved during evolution. The road may be arduous but it is endlessly interesting.

18.7 References

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CHAPTER 19

FUNCTION OF MEMBRANE TRANSPORT SYSTEMS UNDER SALINITY: TONOPLAST

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Abstract

Occupying as much as 90% of the volume of mature cells, the vacuole is a key site for the storage of salt in plants responding to salinity. Vacuolar ion accumulation contributes to both osmotic adjustment and the maintenance of low cytoplasmic ion concentrations. This chapter provides an overview of the alterations in structural and functional properties of vacuolar membrane transporters that occur when plants are exposed to elevated levels of salinity. Particular emphasis is given to the vacuolar-type proton translocating ATPase, a primary-active transporter, which energizes secondary ion transport, and has been investigated extensively with respect to salinity.

19.1 Introduction

One main strategy of plants to cope with high salinity in their environment is the storage of salt in the large central cell vacuole leading to the maintenance of a low cytoplasmic sodium concentration which is prerequisite for the function of a great variety of cytoplasmic enzymes (Flowers *et al.*, 1977). Storage of salt in the vacuole may also be an important strategy for growth and osmotic adjustment under saline conditions, particularly since the vacuole may occupy more than 90% of the volume of mature cells.

Vacuolar salt storage requires the coordinated action of transport proteins present at the tonoplast. Uptake of ions is energized by two primary-active proton pumps, the vacuolar-type proton-translocating adenosine-triphosphatase (V-ATPase) and the vacuolar-type proton-translocating inorganic pyrophosphatase (V-PPase). Both enzymes are able to create an electrochemical proton gradient across the tonoplast, which is used for secondary-active ion uptake mediated by specific transporters, as well as being pivotal to pH homeostasis of the cytoplasm. The main transporter for sodium ions at the tonoplast is a Na^+/H^+ -antiporter, while chloride ions seem to be transported through

channels. Beside these transport proteins directly involved in the uptake of Na^+ and Cl^- into the vacuole, other tonoplastic transporters may play a role in salt accumulation as well as responses of cells to salinity (see Figure 1). In this review we attempt to give an overview about salinity effects on the structural and functional properties of transporters listed above. From the data available so far it is concluded which tonoplast proteins seem to play major roles in vacuolar salt storage.

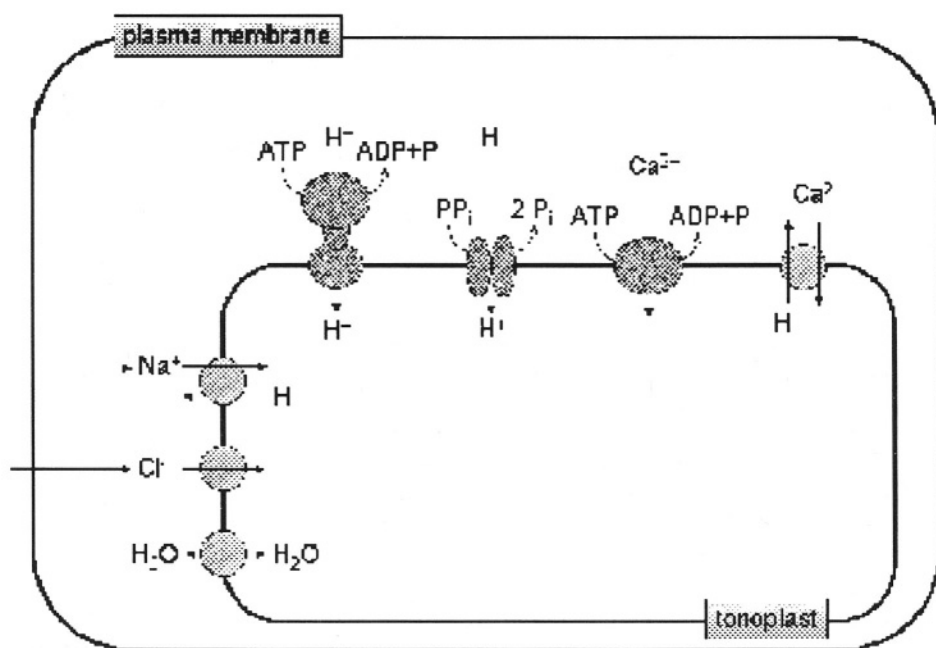


Figure 1. Tonoplast intrinsic proteins possibly involved in vacuolar salt accumulation and storage. The primary-active H^+ -pumps V-ATPase and V-PPase energize the tonoplast for secondary-active transport of ions by proton-translocation into the vacuole using the energy coming from hydrolysis of the γ -phosphate group of ATP or from hydrolysis of inorganic pyrophosphate, respectively. Na^+ is taken up in exchange with protons via a Na^+/H^+ antiporter while Cl^- enters the vacuole electrophoretically driven via a channel. Water channels regulate the transport of water into and out of the vacuole. A Ca^{2+} -ATPase and a $\text{Ca}^{2+}/\text{H}^+$ antiport might influence the cytosolic Ca^{2+} concentration, which could have an impact on regulation of transport activity of the proteins, mentioned above.

19.2 Primary-active Proton Pumps

19.2.1 V-ATPase

19.2.1.1 Subunit composition and holoenzyme structure.

By immunoprecipitation experiments it was shown that the V-ATPase constitutes 6.5% to 24.4% of the total protein present in tonoplast-enriched membrane vesicle fractions

isolated from different plant species (Fischer-Schliebs *et al.*, 1997). The highest amount of V-ATPase (30-35% of total tonoplast protein) was found in the obligate crassulacean acid metabolism (CAM) plant *Kalanchoë daigremontiana* (Klink *et al.*, 1990). The activity of the V-ATPase can be distinguished from other ATPases by its stimulation by chloride, its pH optimum in the range from pH 7-8 and by its inhibition by millimolar concentrations of NO_3^- and nanomolar concentrations of bafilomycin A_1 and concanamycin (for summary see Lüttge and Ratajczak, 1997).

The holoenzyme structure of the V-ATPase is rather complex (for reviews, see Sze *et al.*, 1992, Lüttge and Ratajczak, 1997). In some plant species up to 10 different subunits have been identified. This is close to the number of 15 subunits found to be elements of the yeast and mammalian V-ATPase holoenzyme (Nelson and Klionsky, 1996) and it can be expected that subunits homologous to the 5 subunits which have not been found in plants up to now will be identified within the next few years.

Electron microscopical investigations (Klink and Lüttge, 1991, Lee Taiz and Taiz, 1991) revealed that the structure of the V-ATPase is very similar to the F-type ATP synthase (F-ATPase) present in the inner mitochondrial membrane and in thylakoid membranes, containing a membrane integral domain (V_o) and a membrane peripheral domain (V_1) which can be visualized by freeze-fracture electron microscopy and negative-stain electron microscopy, respectively. V_1 exhibits a head-and-stalk structure. The largest subunits A (catalytic subunit; ca. 70 kDa) and B (regulatory subunit; ca. 60 kDa) are located in multiple copies in the head while the central stalk contains subunits C (ca. 40 kDa), D (30-35 kDa), E (25-30 kDa) and subunit G (14 kDa) the latter of which was recently found to be part of the plant V-ATPase (Rouquié *et al.*, 1998). Main components of V_o are several copies of subunit c (16 kDa; proteolipid) which is suggested to be involved in proton translocation across the tonoplast. In addition to subunit c, V_o contains polypeptides exhibiting molecular masses of 95-115 kDa, 30-32 kDa, 13 kDa and 12 kDa (for review, see Lüttge and Ratajczak, 1997).

From early electron microscopical observations there was only evidence for a single central stalk connecting the V_o -domain with the V-ATPase head. However, recently a second peripheral stalk of the Na^+ -translocating V-ATPase of *Clostridium fervidus* has been discovered (Boekema *et al.*, 1997). Re-investigation of the F-ATPase (Böttcher *et al.*, 1998) using high-resolution electron microscopy and more sophisticated methods of image analysis also led to the identification of a peripheral stalk. Wilkens and Forgac (1998) reported evidence for a subunit located asymmetrically on one side on the top of V_1 of the proton translocating V-ATPase, which is a good candidate to be part of a second peripheral stalk. In analogy to the actual functional model of the F-ATPase, the peripheral stalk of the V-ATPase is discussed as a potentially important part of a rotor-stator system essential for the coupling of ATP hydrolysis and proton transport (see below and Junge *et al.*, 1997).

19.2.1.2 V-ATPase activity changes in plants responding to salinity.

The first hints for differential modulation of ATPase activity by salinity in roots of halophytes and non-halophytes came from experiments using the halophyte *Atriplex nummularia* (Braun *et al.*, 1986) and *Gossypium hirsutum* (Hassidim *et al.*, 1986). While in membrane vesicles isolated from the halophyte ATP-dependent H^+ -transport activity was 2-fold higher in preparations from salt-treated plants compared to controls,

salinity did not change H^+ -transport activity of vesicles isolated from the non-halophyte. However, in the studies mentioned above ATPase activity changes seem to be mainly due to the P-ATPase of the plasma membrane since ATPase activity of membrane vesicles from *At. nummularia* was not significantly inhibited by NO_3^- and in *G. hirsutum* vesicles NO_3^- inhibition was around 25% of total ATP-hydrolysis activity. Studies on tonoplast-enriched membrane vesicle fractions isolated from leaf cells of the halophyte *Mesembryanthemum crystallinum* clearly showed that H^+ -transport and ATP-hydrolysis activity of the V-ATPase was significantly increased in salt treated plants (Struve and Lüttge, 1987 a, b, Bremberger *et al.*, 1988). However, since salt-treatment of *M. crystallinum* plants leads to a change of metabolism from C_3 -photosynthesis to Crassulacean acid metabolism (CAM) it was difficult to distinguish whether the increase in V-ATPase activity was due to salinity or the metabolic switch. By monitoring the time course of V-ATPase activity and amount during salt-treatment it was possible to show that the increase in V-ATPase activity related to total tonoplast protein found in the studies mentioned above is mainly an effect of salinity (Ratajczak *et al.*, 1994). There are several other reports about increase of V-ATPase substrate hydrolysis activity due to salinity in the literature (cytochemical determination of ATP-hydrolysis activity of *Lycopersicon esculentum* roots, Sanchez-Aguayo *et al.* (1991); tonoplast-enriched membrane vesicles from *Sorghum bicolor* roots, Koyro *et al.* (1992) and *Vigna radiata* roots, Nakamura *et al.* (1992)). In microsomal fractions and tonoplast-enriched membrane vesicles isolated from salt-treated *Hordeum vulgare* roots, H^+ -transport activity was increased compared to controls (Matsumoto and Chung, 1988). Salinity-dependent increase in V-ATPase activity is not restricted to the whole plant system. Reuveni *et al.* (1990) demonstrated an increase in ATP-hydrolysis activity in preparations of tonoplast-enriched membrane vesicles from cultured cells of *Nicotiana tabacum* grown at 428 mM NaCl and Vera-Estrella *et al.* (1999) found an increase of V-ATPase hydrolytic activity in tonoplast-enriched membrane vesicles from suspension cultured cells of *M. crystallinum* grown at 100-400 mM NaCl. However, it has to be mentioned that mild salt treatment did not change ATP-hydrolysis activity in microsomal fractions isolated from cells of *Daucus carota* (50 mM NaCl; Colombo and Cerana, 1993) and *Acer pseudoplatanus* (80 mM NaCl; Zingarelli *et al.*, 1994). No change in ATP-hydrolysis activity was observed in tonoplast-enriched membrane vesicles isolated from roots of *Spartina townsendii* (Koyro *et al.*, 1992) and *Helianthus annuus* (Ballesteros *et al.*, 1997).

An important factor which is often overlooked is that the V-ATPase exhibits two different activities, i.e. ATP-hydrolysis and H^+ -transport activity, and changes in one may not necessarily reflect changes in the other. In most of the studies mentioned above only one of these activities was monitored when looking for a response to salt stress. Studies of both V-ATPase activities using the same batches of tonoplast-enriched membrane vesicle fractions from *He. annuus* roots (Ballesteros *et al.*, 1996) and *Ho. vulgare* leaves (Mariaux *et al.*, 1997) revealed that salt stress increased H^+ -transport activity while ATP-hydrolysis was not significantly changed. This interesting finding cannot be explained by a simple increase in V-ATPase protein amount and thus leads us to the next section addressing the question: Which factors may influence V-ATPase activity changes caused by salinity?

19.2.1.3 Causes of increased activity under salinity.

The simplest explanation for a salinity induced increase in V-ATPase activity is the increase of active V-ATPase protein in the membrane vesicle preparation studied. Salinity-induced changes of the *M. crystallinum* leaf V-ATPase amount has been studied using various techniques. On silver stained gels the staining intensity of V-ATPase subunits increased with the onset of salt treatment (Bremberger and Lüttge, 1992; Rockel *et al.*, 1994). By radial immunodiffusion using specific antibodies against the V-ATPase holoenzyme it could be shown that V-ATPase protein amount related to total tonoplast protein increases by a factor of 2.5 during the first 8 days of treatment with 400 mM NaCl and stayed at this high level under prolonged salt treatment (Ratajczak *et al.*, 1994). When salt was removed from the root medium after 8 days V-ATPase amount decreased within 48 h to the value present in the tonoplast of control plant leaves indicating a rapid turnover of the V-ATPase holoenzyme (Ratajczak *et al.*, 1994). Higher amounts of V-ATPase in salt-treated *M. crystallinum* plants compared to controls were also detected by quantitative immunoprecipitation of the V-ATPase holoenzyme using antibodies against subunit A and immuno-electron microscopy of tonoplast vesicles (Ratajczak *et al.*, 1995). Western blot analysis revealed that subunit E (26 kDa) protein amount increases due to salt-treatment in protein extract from young and old leaves of *M. crystallinum* (Dietz and Arbing, 1996). Taken together, salt-treatment leads to an increase in V-ATPase hydrolysis activity and V-ATPase protein amount in *M. crystallinum* leaves. Thus, the specific ATP-hydrolysis activity (rate of substrate hydrolysis per V-ATPase) is unaltered and the increase in V-ATPase hydrolytic activity related to total tonoplast protein (which often has been misnomered 'specific activity') is due to an increase of V-ATPase protein related to total tonoplast protein.

An increased abundance of V-ATPase protein is not necessarily associated with increased V-ATPase activity observed with salinity. Hurkman *et al.* (1988) used two-dimensional polyacrylamide gel electrophoresis to compare the polypeptide pattern of membrane vesicle fractions enriched in Golgi apparatus and tonoplast vesicles from control and salt-treated *Ho. vulgare* roots. Silver staining intensities and radioactive labeling of V-ATPase subunits A and B revealed only slight increase in the polypeptides due to salinity. Western blot analyses (DuPont, 1992) supported the conclusion that V-ATPase protein amounts do not increase in the roots of *Ho. vulgare* plants responding to salinity, despite a 2-fold increase in H^+ -transport activity. In salt-adapted cells of *N. tabacum*, V-ATPase A subunit protein content decreased 4-fold, as compared to control cells (Reuveni *et al.*, 1990).

The immunological data in the above cited work have relied on poly-clonal antisera, which have been assumed to recognize all forms of the V-ATPase (Reuveni *et al.*, 1990). Based on the extensive degree of sequence homology for the various V-ATPase subunits amongst different species (Gogarten *et al.*, 1992, Kibak *et al.*, 1992), this would seem to have been a reasonable assumption. However it has been recently demonstrated that antisera to the V-ATPase holoenzyme from *K. diagraemontiana* exhibit differential immunological cross-reactions to subunits of the V-ATPase from different plant species (Fischer-Schliebs *et al.*, 1997). Therefore some poly-clonal antisera may underestimate the amount of total V-ATPase protein present in a given sample. Thus some of the interpretations about whether salinity is or is not associated

with an increase in the amount of V-ATPase protein should be regarded cautiously until a better understanding is gained about the ability of these antisera to discriminate between different forms of the enzyme.

But what about the finding that in tonoplast vesicles from *He. annuus* roots (Ballesteros *et al.*, 1996) and *Ho. vulgare* leaves (Mariaux *et al.*, 1997) ATP-hydrolysis activity remains unchanged under salinity while **H⁺-transport** activity is increased? In both studies it was demonstrated by Western blot analyses that the amount of V-ATPase subunits A and B was not affected by salt treatment. This is in agreement with the unaltered ATP-hydrolysis activity. However, it does not explain the higher rates of **H⁺-transport** under salinity. The latter might be due to a change of the properties of the tonoplast influencing proton permeability and/or of **V_o-domain** structure leading to a change of coupling ratio ($n \text{ H}^+_{\text{transported}}/\text{ATP}_{\text{hydrolyzed}}$) of the V-ATPase. Unfortunately, neither the lipid composition nor the amount of the proton translocating subunit c were investigated. On the other hand, Mariaux *et al.* (1997) inspected the diameter of intramembrane particles (IMPs) on freeze-fracture replicas of *Ho. vulgare* tonoplast vesicles, which mainly represent the **V_o-domain** of the V-ATPase. The size distribution of IMPs was identical in samples from controls and salt-treated plants indicating that there was no drastic change in the structure, and thus in the subunit composition of **V_o**. In the same study a different result was obtained inspecting V-ATPase activity and IMPs diameters of tonoplast vesicles isolated from *Kalanchoë blossfeldiana* with different levels of CAM expression. Preparations from plants exhibiting a higher degree of CAM exhibited a lower relative coupling ratio (**H⁺-transport** activity and ATP hydrolysis activity were higher by factors of 2.4 and 3.2, respectively) and larger IMPs compared to preparations from plants exhibiting a lower degree of CAM. A similar correlation of coupling ratio and IMP diameter has been found in *M. crystallinum*. Investigation of IMPs of tonoplast vesicles from *M. crystallinum* showed that **V_o** diameter in salt-treated CAM-plants and well-watered **C₃-plants** is 8.5-9.1 nm and 6.5-7.3 nm, respectively (Klink and Lüttge, 1992, Rockel *et al.*, 1994). Since analysis of silver-stained gels and Western blots immunostained with specific V-ATPase antisera revealed that after salt-treatment staining intensity of subunit c increases to a higher extent than staining intensities of the other V-ATPase subunits (Bremberger and Lüttge, 1992, Ratajczak *et al.*, 1994, Rockel *et al.*, 1994, Berndt *et al.*, 1999) it was proposed that the increase in **V_o-diameter** might be due to a higher copy number of subunit c per holoenzyme. Recently, it was shown by simultaneous measurements of **H⁺-transport** activity and ATP-hydrolysis activity that the relative coupling ratio of the *M. crystallinum* CAM-V-ATPase is lower than the *M. crystallinum* **C₃-V-ATPase** coupling ratio by a factor of 1.3 (Berndt *et al.*, 1999). The difference in coupling ratio seems to be due to changes of the V-ATPase since passive proton leakage was identical in vesicles isolated from salt-treated plants and controls (Berndt *et al.*, 1999). Similar results have been reported by Vera-Estrella *et al.* (1999) investigating tonoplast-enriched membrane vesicles isolated from cultured cells of *M. crystallinum*. Although the authors do not comment on it, an approximately 1.6-fold lower coupling ratio of the V-ATPase from salt-treated cells compared to controls can easily be calculated from the presented rates of proton-transport activity and ATP-hydrolysis activity. These findings could be interpreted in the view of the actual functional model of F-ATPase and V-ATPase (Junge *et al.*, 1997). According to this model the F-ATPase contains a rotor made up by a revolver-like ring of membrane integral c-subunits coupled to the **γ-subunit** (which is

homologue to the V-ATPase subunit D, Kluge *et al.*, 1999). The rotor carries out Brownian rotational fluctuations relative to the stator, which is made up by the other F-ATPase subunits. An aspartyl residue located in one of the two membrane spanning domains of the F-ATPase subunit c quite in the center of the lipid bilayer is suggested to be of major importance for coupling of ATP-hydrolysis or –synthesis and proton transport. In its deprotonated state it is reflected by stator subunits. Only after protonation of the aspartyl residue, the ring of c-subunits is able to move one step further. The coupling of the rotating ring of c-subunits with subunit γ leads to conformational changes in the enzyme head influencing the conformation of subunits α and β (which are homologue to V-ATPase subunits B and A) and resulting in ATP hydrolysis or synthesis. Due to the high degree of homology between the F-ATPase and V-ATPase, the functional model of the F-ATPase might also be true for the V-ATPase. The mode of action described above offers an interesting mechanism for regulation of the coupling ratio of the V-ATPase. Modification of the number of c-subunits present in V_o under certain environmental conditions like high salinity, creating a steep NaCl concentration gradient between the cytoplasm and the vacuolar lumen as is the case in *M. crystallinum*, would act like a gear shift mechanism modifying the coupling ratio. A lower coupling ratio would benefit proton transport into the vacuole, since the amount of energy available for transport of a single proton would be higher. However, up to now changes in V_o -diameter have only been observed in plants with inducible CAM and might be more CAM-related than salinity-related. Actually, in the C_3 -plant *Ho. vulgare* a higher coupling ratio was found in preparations from salt-treated plants (Mariaux *et al.*, 1997). Leaf cell sap Na^+ and Cl^- concentrations of the plants analyzed were around 250 mM which is roughly half of the concentrations reached in *M. crystallinum* leaves. Thus, the NaCl-concentration gradient across the tonoplast is lower in *Ho. vulgare*. Under these circumstances an increase in coupling ratio could be sufficient to energize Na^+ and Cl^- uptake into the vacuole without changing the V-ATPase protein amount. A possible explanation for the higher coupling ratio in preparations from salt-treated *Ho. vulgare* plants exhibiting identical IMP diameter as controls could be the incorporation of different c-subunit isoforms in V_o which might differ in the number of protonable negatively charged amino acid side chains (see below). All plant c-subunits sequenced so far have a high degree of sequence similarity to the yeast *vma3* gene the gene product of which is essential for V-ATPase function (Stevens and Forgac, 1997). In yeast two additional proteolipid genes have been identified: *vma11* (subunit c') and *vma16* (subunit c''). Subunits c and c' exhibit a high degree of sequence similarity, while subunit c'' differs from subunit c and c'. In contrast to the four membrane spanning domains of c and c', subunit c'' contains five membrane spanning domains. According to Stevens and Forgac (1997) all three polypeptides are essential for the function of the yeast V-ATPase. Up to now it was not shown that c' and c'' are elements of the plant V-ATPase. If they are, however, changes in the c, c' and c'' composition of V_o could also lead to changes in enzyme properties.

Several other mechanisms have been suggested to alter plant V-ATPase activity, i.e. phosphorylation of V-ATPase subunits (Zocchi, 1985, Scherer *et al.*, 1988, Scherer and Stoffel, 1987, Garbarino *et al.*, 1991, Martiny-Baron *et al.*, 1992), modification of the redox state of the enzyme by oxidation and reduction of essential sulphhydryl groups present in V-ATPase subunits A and B (Hager and Lanz, 1989, Yamanishi and Kasamo, 1992) or simply changes in the availability of the V-ATPase substrate MgATP (Dietz *et*

al., 1998). However, none of these mechanisms has been proven to play a role in the response of the V-ATPase to salinity. Another factor influencing V-ATPase activity is the lipid environment of the enzyme. Removal of lipids from purified V-ATPase resulted in inactivation of the enzyme (Yamanishi and Kasamo, 1993). Enzyme activity could be restored by addition of phospholipids indicating that the phospholipids in the liquid crystalline phase are necessary for V-ATPase activity. Since it has been reported that salinity modifies the tonoplast lipid composition in some species (see below) these changes could influence V-ATPase activity.

The occurrence of the polypeptides D_i (32 kDa) and E_i (28 kDa) associated with the V-ATPase in salt-treated *M. crystallinum* plants in the CAM-state might have an impact on V-ATPase properties. It has been shown that D_i is a proteolytically processed subunit B which is present *in vivo* (An Zhigang *et al.*, 1996). Recently, it was demonstrated that the amount of D_i in tonoplast vesicle preparations shows diurnal fluctuations, D_i amount increases from the late afternoon to midnight and subsequently decreases again (Berndt *et al.*, 1999). By gas exchange measurements it was shown that phase IV of CAM (stomatal opening and uptake of CO_2 in the afternoon) was not expressed under the growth conditions used (Ratajczak *et al.*, 1998 b). Thus, it is likely that after decarboxylation of the nocturnally accumulated malate (at about 2 p.m.), internal CO_2 concentration decreases, while due to photosynthetic electron transport, internal O_2 concentration increases leading to higher rates of Mehler reaction and oxidative stress. Evidence for elevated oxidative stress in salt-treated *M. crystallinum* plants comes from the finding that superoxide dismutase activity is higher in salt-treated plants compared to controls (Miszalski *et al.*, 1997). Oxygen radicals have been shown to modify amino acid side chains of proteins including the methionine side chain (Davies *et al.*, 1987). Since the *M. crystallinum* subunit B is cleaved at the C-terminal side of Met¹⁹² the formation of D_i could be linked to oxidative stress. Salt-treatment of *Citrus sinensis* leads to proteolysis of subunit A in the tonoplast of leaf cells (Bañuls *et al.*, 1995) which might also be linked to the occurrence of salinity-induced oxidative stress. Future work has to show if stress induced proteolytic processing of V-ATPase subunits leads only to higher V-ATPase turnover, or has an impact on V-ATPase properties. In the case of *M. crystallinum* tonoplast vesicles, similar specific ATP-hydrolysis activities of the C_3 - and the CAM-V-ATPase were determined indicating that the occurrence of D_i does not affect enzyme activity (Ratajczak *et al.*, 1994). However, the D_i containing V-ATPase from salt-treated CAM plants is less sensitive to dissociation by high salt concentrations (Lüttge *et al.*, 1995) and conformational changes induced by detergents (Ratajczak, 1994).

19.2.1.4 Impact of salinity on the regulation of V-ATPase gene expression.

While structural modifications may be in part responsible for the elevated V-ATPase activity associated with the response of plants to salinity, there is also evidence that altered gene expression plays a role. There have been numerous observations of increased accumulation of transcripts for the V-ATPase in plants and cells responding to salinity. Narasimhan *et al.* (1991) first reported on an increased accumulation of A-subunit message (2 to 4-fold) in salt adapted *N. tabacum* cells responding to NaCl, but this increase was only observed during the mid-linear stage of the growth cycle. Increased accumulation of A-subunit message in response to salinity has also been observed in *L. esculentum* (Binzel, 1994, 1995, Binzel and Dunlap, 1995), and *Beta*

vulgaris (Kirsch *et al.*, 1996, Lehr *et al.*, 1998) but not *M. crystallinum* (L  w *et al.*, 1996). Increased accumulation of subunit c message has also been reported in response to salinity in *Arabidopsis thaliana* (Perera *et al.*, 1995), *B. vulgaris* (Kirsch *et al.*, 1996, Lehr *et al.*, 1998), and *M. crystallinum* (L  w *et al.*, 1996, Tsiantis *et al.*, 1996).

In *L. esculentum* increased accumulation of subunit A message was found in fully expanded leaves, but not in the youngest leaves or in roots (Binzel, 1995). Lehr *et al.* (1998) found that in a diploid cultivar of *B. vulgaris*, NaCl brought about an increased accumulation of V-ATPase subunit A and c message in roots, although no increase had previously been observed in the roots of a tetraploid cultivar of *B. vulgaris* (Kirsch *et al.*, 1996). These data highlight the fact that NaCl-induced alterations in message accumulation are superimposed upon pre-existing developmental and tissue/organ specific programs of gene expression. Differences in constitutive levels of V-ATPase expression may in part dictate when and where it is necessary to elevate the pre-existing level of V-ATPase expression, to meet the demands imposed by salinity. In the case of *L. esculentum*, constitutive levels of A-subunit message accumulation are quite high in roots and unexpanded leaves (Binzel, 1995), and transcriptional activation of genes encoding the V-ATPase subunits may be unnecessary. Some of these observations may also point to differences in the demand for proton gradient formation and maintenance in different tissues, or at different stages of development, as plants respond to salt.

An interesting question has been whether or not the various subunits undergo coordinate genetic regulation, and if salinity would modulate gene expression in concert. Initially it appeared as if this might not be the case, as results from *M. crystallinum* indicated that there was non-coordinate regulation of the A-, B- and c-subunits in response to salinity. Subunit c message increased dramatically in response to several hours of salt stress, while levels of A and B messages remained fairly constant (L  w *et al.*, 1996). On the other hand, it could be demonstrated that long-term salt stress (12 days) increased the message amount for subunits A, B and c by factors of 2, 12 and 5, respectively (Rockel *et al.*, 1998). This could be explained by the assumption that levels of A and B message increase after a short lag phase compared to c message. It is an interesting finding that subunit B and c messages are increased much more pronouncedly than the increase of subunit A message. As mentioned above changes of the *M. crystallinum* V-ATPase at the protein level are mainly due to changes of subunits B (proteolytic processing and occurrence of the D_i polypeptide) and c (increase in V_o-diameter which is possibly correlated with a higher amount of c-subunit copies per V-ATPase holoenzyme). Thus, pronounced changes of B and c message might reflect the requirement for a higher rate of protein synthesis of these subunits compared to subunit A. Additional evidence for non-coordinated expression of *M. crystallinum* V-ATPase subunits came from analysis of subunit E message amount, which did not change during the first 48 h of salt stress (Dietz and Arbing, 1996). However it appears that *M. crystallinum* may be somewhat unique in this respect, because other studies have suggested a coordinate induction of expression by salinity for A- and c-subunits in *B. vulgaris* and *D. carota* (Kirsch *et al.*, 1996, Rausch *et al.*, 1996). Comparative analysis of a promoter from an A-subunit gene from *B. vulgaris* and one from a c-subunit (Lehr *et al.*, 1999) suggest a similar degree of stimulation by salinity (70% and 57%, respectively), although relative promoter strength varied by a factor of 1.7 (c over A).

19.2.1.5 *The role of isoforms*

One of the factors that has yet to be resolved, and which will probably significantly improve our understanding of the participation of the V-ATPase in the response of plants to salinity is the role of isoforms. All plant V-ATPase subunits investigated so far seem to be present in different isoforms (for review, see Lüttge and Ratajczak, 1997). Small gene families of subunit c have been detected in various species (*Ar. thaliana*, Perera *et al.* (1995); *Avena sativa*; Lai *et al.* (1991); *G. hirsutum*, Hasenfratz *et al.* (1995); *M. crystallinum*, Löw *et al.* (1996); *Zea mays*, Viereck *et al.* (1996)). These multiple isoforms of the c-subunit seem to be nearly identical in the coding regions, but differ significantly in the non-coding regions. Two or more isoforms of the A-subunit have been observed in all plant species except *Ar. thaliana* (Starke and Gogarten, 1993) and *B. vulgaris* (Kirsch *et al.*, 1996) where only one gene has been observed. Less information is available about potential isoforms for the B-subunit, but based on immunological evidence, it would appear that there may be two isoforms of this subunit as well (Randall and Sze, 1989, Hurkman *et al.*, 1988).

Using gene specific probes, it has been possible to document that at least some of these isoforms are differentially expressed with respect to tissue localization and developmental regulation. Hasenfratz *et al.* (1995) observed in *G. hirsutum* that c-subunit isoforms accumulated differentially during development, and in different tissues. Unfortunately, most of the studies looking for evidence of increased message accumulation in response to salinity have been performed with probes that do not discriminate between different isoforms of the V-ATPase subunits. In *M. crystallinum*, the *Vmac1* transcript was found to be preferentially expressed in leaves, but it was not clear from the results that this was the only transcript that was accumulating in response to salt (Tsiantis *et al.*, 1996). It is tempting to speculate that the role of these different isoforms, and the genes that encode them, is primarily to modulate expression and targeting, rather than to contribute to altered enzyme properties. One can envision that certain isoforms would be responsible for the constitutive or "house-keeping" expression of the V-ATPase, while others would be designed to respond to specific environmental stimuli. However, despite the observation that all sequenced c-subunits exhibit a high degree of homology of nucleotide sequences, apparently there are at least minor variations at the protein level as differences can be detected using specific antisera (Fischer-Schliebs *et al.*, 1997). These different structural features may have an impact on polypeptide properties or enzyme function (e.g. alterations in coupling ratios discussed previously). It will not be possible to reach a conclusion regarding what role if any the existence of different isoforms plays until it is possible to begin characterizing specific V-ATPase holoenzymes with respect to their subunit and isoform composition, and then to be able to examine a variety of biochemical and physiological parameters of the enzyme to discern variations in properties and function.

19.2.1.6 *How does salinity regulate V-ATPase gene expression?*

Endogenous oscillation was demonstrated for V-ATPase subunit c transcript accumulation (Rockel *et al.*, 1997). This is similar to the behavior of the transcript for highly regulated plant proteins (e.g. the small subunit of ribulose-bis-phosphate carboxylase/oxygenase (Kloppstech, 1985) or chlorophyll a/b-binding light harvesting proteins (Millar and Kay, 1991)). Thus, it can be suspected that also the V-ATPase c-

subunit and maybe other V-ATPase subunits might be highly regulated proteins. However, although genes encoding subunits of the V-ATPase have been cloned from many species (for review, see Lüttge and Ratajczak, 1997), relatively little is known regarding the features of the promoters controlling these genes, and how environmental stimuli such as salinity modulate their expression.

There are some hints that the expression of V-ATPase genes and modification of the V-ATPase protein could be regulated by phytohormones. Kasai *et al.* (1993) found that treatment of *Ho. vulgare* plants with abscisic acid (ABA) significantly increased V-ATPase proton transport activity of tonoplast vesicles isolated from root tissue, while the proton transport activity was not affected by treatment with the cytokinin 6-benzyladenine. *In vivo* treatment of *Ho. vulgare* with the P-ATPase inhibitor vanadate led to an increase in root internal ABA concentration and to an increase in ATP-dependent H^+ -transport activity of tonoplast vesicles (Kasai *et al.*, 1994). In this respect it is interesting that levels of ABA and the ethylene precursor aminocyclopropane-1-carboxylic acid (ACC) increased in *Citrus* seedlings after salt shock (Gómez-Cadenas *et al.*, 1998). After induction of senescence by treatment of *Ho. vulgare* leaves with methyljasmonate, changes of the subunit pattern of the V-ATPase and of the pH-optimum of ATP-hydrolysis activity could be detected (Ratajczak *et al.*, 1998 a). In a recent review Bressan *et al.* (1998) pointed out the possible important role of Ca^{2+} for the regulation of V-ATPase expression. It is well known that an increase in Ca^{2+} concentration reduces inhibitory effects caused by salinity (Läuchli, 1990). Since the role of Ca^{2+} as a second messenger in biological systems is well established, salinity-induced changes in Ca^{2+} concentration could affect signal transduction pathways involving Ca^{2+} . A possible candidate for a calcium sensing protein is the *Ar. thaliana* SOS3 gene product (Liu and Zhu, 1997), mutation of which leads to an increase in salt-sensitivity of the plant. SOS3 exhibits high sequence homology to the yeast calcineurin B subunit and a neuronal Ca^{2+} sensor. Tonoplast proteins which might be involved in the regulation of the cytoplasmic Ca^{2+} concentration which could be part of a signal transduction chain regulating gene expression are the primary-active Ca^{2+} -ATPase (Pfeiffer and Hager, 1993, Askerlund, 1997) and a Ca^{2+}/H^+ antiport (Blumwald, 1987).

Recently Lehr *et al.* (1999) have begun to examine promoters from A- and c-subunit genes from *B. vulgaris*, to begin addressing this question. Prior to this work, the only other information available on the promoters from a plant V-ATPase gene, was the work of Struve *et al.* (1990), on the promoter for a gene encoding subunit A from *D. carota*. One of the important findings of the work of Lehr *et al.* (1999), is the observation of greater promoter activity in the presence of NaCl, supporting the inference that increased transcript abundance associated with salinity is a result of transcriptional activation. Features common to these two promoters are relatively long 5'UTRs (also observed in other V-ATPase genes), CT-rich regions in the 5'UTRs, and G-box motifs (Lehr *et al.*, 1999). The CT-rich regions may provide some clues to structural elements involved in salt-induced regulation, since a similar feature has been noted in two other salt-inducible genes (Cushman and Bohnert, 1992, Tsiantis *et al.*, 1996). At this time, it is uncertain whether the G-boxes identified in these promoters function in an ABA-mediate pathway of NaCl response. In *L. esculentum*, ABA does not appear to mediate the NaCl-induced up-regulation of the A-subunit (Binzel and Dunlap, 1995), while in *M. crystallinum*, it has been suggested that the *Vmac1* gene is

up-regulated by ABA (Tsiantis *et al.*, 1996). Considering the evolving picture regarding the complexity of ABA-dependent and independent pathways of stress-induced gene activation (Ishitani *et al.*, 1997), it is highly probable that V-ATPase genes, which are responsible for house-keeping as well as stress response functions, will exhibit a complex pattern of regulatory features. Definitive conclusions regarding the manner in which environmental stimuli are transduced to transcriptional activation will require detailed functional analysis of promoters. And as suggested by the evidence on transcript accumulation patterns, there is likely to be a convergence of regulatory mechanisms, representing not only environmental stimuli, but developmental and cell or tissue specific cues as well.

19.2.2 V-PPase

19.2.2.1 *Properties and possible functions of the V-PPase.*

In comparison to the V-ATPase the structure of the V-PPase appears to be rather simple. The functional form of the V-PPase is proposed to be a homo-dimer of a polypeptide exhibiting an apparent molecular mass of ranging from 64-81 kDa when isolated from different plant species (for reviews, see Leigh *et al.*, 1994, Zhen *et al.*, 1997). Cloning and sequencing of the V-PPase of *Ar. thaliana* (Sarafian *et al.*, 1992), *Ho. vulgare* (Tanaka *et al.*, 1993), *B. vulgaris* (Kim *et al.*, 1994), *N. tabacum* (Lerchl *et al.*, 1995) and *Oryza sativa* (Sakakibara *et al.*, 1996) revealed a molecular mass of 79-81 kDa (760-775 amino acids) for the V-PPase monomer. However, all the functional features characteristic for the function of the V-ATPase, i.e. a substrate binding domain, a proton translocating domain and binding domains of effectors are also present in the V-PPase. Estimation of the V-PPase amount indicates that the enzyme constitutes up to 5-10% of the total tonoplast protein (Maeshima and Yoshida, 1989, Ratajczak *et al.*, 1995), and thus is present in the tonoplast in a lower protein amount than the V-ATPase. On the other hand, the amount of active pump molecules of the V-ATPase and the V-PPase seems to be in the same order of magnitude. Taking into account a molecular mass of approximately 750 kDa and 160 kDa and a relative amount of 30% and 10% of total tonoplast protein for the V-ATPase holoenzyme and the V-PPase dimer, respectively, one could calculate relative abundance numbers (molecular mass divided by relative amount of total tonoplast protein) for both enzymes. The value of 16 which can be calculated in this way for the functional V-PPase dimer is very close to the value of 25 for the V-ATPase holoenzyme. This might explain why the V-PPase is able to generate an electrochemical proton gradient across the tonoplast which is similar or greater than the gradient formed by the action of the V-ATPase at the same membrane (Pope and Leigh, 1987, Maeshima and Yoshida, 1989, Johannes and Felle, 1990, Rea *et al.*, 1992). This indicates that the V-PPase could play a major role in tonoplast energization.

19.2.2.2 *V-PPase responses under salinity.*

Since the tonoplast contains two primary-active proton pumps, i.e. the V-ATPase and the V-PPase, it is favorable to study salt stress effects on both enzymes to evaluate which proton pump is more important for the plant cell to cope with high cytosolic salt concentrations. This was done in the case of *M. crystallinum* using tonoplast-enriched membrane vesicles isolated from leaves during salt-induced C₃-CAM shift. It turned out that in contrast to the V-ATPase (see above) V-PPase hydrolytic activity (Bremberger *et*

al., 1988, Bremberger and Lüttge, 1992) and amount (Rockel *et al.*, 1994) decreased with prolonged salt treatment. However, similar effects were observed when CAM was expressed during aging of the plants in the absence of salt in the root medium (Rockel *et al.*, 1994). Thus, decrease in V-PPase activity and amount seem to be correlated to CAM expression and not to salinity *per se*. On the other hand experiments performed with another **C₃-CAM** intermediate plant, *K. blossfeldiana*, revealed that the results obtained with *M. crystallinum* cannot be generalized. In *K. blossfeldiana* CAM is induced by a change in the length of the photoperiod. Under these conditions both V-PPase activity and protein amount are increased during the **C₃-CAM** shift (Fischer-Schliebs *et al.*, 1998), indicating that downregulation of the V-PPase is not a general feature of CAM.

Unfortunately, salt treatment of **C₃-plants** has not lead to unequivocal results either. Salt-treatment of the halophyte *Suaeda maritima* did not change V-PPase substrate hydrolysis activity and properties (Leach *et al.*, 1990 a). Similar results have been obtained after salt-treatment of cultured *N. tabacum* cells (Reuveni *et al.*, 1990). On the other hand, salt-treatment led to a decrease in V-PPase activity in root cells of *V. radiata* (Nakamura *et al.*, 1992) while in cultured cells of *D. carota* (Colombo and Cerana, 1993) and *Ac. pseudoplatanus* (Zingarelli *et al.*, 1994) V-PPase activity was induced by salinity. An interesting finding was published by Ballesteros *et al.* (1996). The authors found that salt treatment of *He. annuus* led to a slight increase of root cell V-PPase substrate hydrolysis activity in tonoplast-enriched membrane vesicle fractions while **H⁺-transport** activity was increased more pronouncedly. This is a hint for a modulation of the coupling ratio of the V-PPase under certain metabolic conditions (see above for discussion of modulation of the coupling ratio of the V-ATPase).

19.3 Na⁺/H⁺ Antiport

The compartmentalization of Na⁺ into the vacuoles of plants and cells exposed to salinity has been well documented (Yeo, 1981, Binzel *et al.*, 1988; Chapter 8). Vacuolar sequestration of accumulated Na⁺ reduces potential toxicity in the cytosol and contributes to the osmotic potential of the cell. The transport of Na⁺ into the vacuole has long been presumed to occur through a Na⁺/H⁺ antiport system which utilizes the proton electrochemical gradient generated by the V-ATPase and/or V-PPase, to drive Na⁺ uptake across the tonoplast and into the vacuole (reviewed in Barkla *et al.*, 1994, Rausch *et al.*, 1996, Barkla and Pantoja, 1996). The presence of such an antiport system in plants was first documented by Blumwald and Poole (1985), in tonoplast enriched membrane vesicles from storage tissue of *B. vulgaris*. Electroneutral Na⁺-dependent H⁺-efflux exhibiting Michaelis-Menten kinetics with respect to Na⁺-concentration and competitive inhibition by amiloride was observed and attributed to a Na⁺/H⁺ antiporter on the tonoplast. While the activity of this transporter has been readily observable in tonoplast-enriched membrane vesicle preparations from numerous plants, the biochemical identification and characterization of the transporter has been elusive.

19.3.1 INDUCTION BY SALINITY

Studies documenting the induction of Na^+/H^+ antiport activity in plants exposed to salt supported a role for this transporter in salinity tolerance. The V_{\max} of the tonoplast Na^+/H^+ antiporter in *B. vulgaris* suspension-cultured cells treated with NaCl was approximately twice that measured in control cells, while the K_m remained unchanged (Blumwald and Poole, 1987). Similar results were obtained when the tonoplast Na^+/H^+ antiporter from leaves of *M. crystallinum* (Barkla *et al.*, 1995) was examined. Both these studies suggest increased Na^+/H^+ antiport activity upon exposure to salt above constitutive levels of activity, presumably through an increased abundance of antiporter molecules. However results from other studies point to *de novo* tonoplast Na^+/H^+ antiport activity via activation of pre-existing antiporter molecules upon exposure to salt. Garbarino and DuPont (1988) found no constitutive Na^+/H^+ activity in the roots of *Ho. vulgare*, but observed a rapid ($t_{1/2}=15$ min) induction of activity which was independent of protein synthesis. In *He. annuus* no constitutive activity was observed, but activity was found in the roots after plants had been treated with NaCl. The K_m of this activity in the roots of plants treated with 150 mM NaCl was lower than that measured in the roots of plants treated with 75 mM NaCl, while the V_{\max} for the two remained unchanged (Ballesteros *et al.*, 1997). If one generalizes from this limited set of data, it would seem as if naturally salt-tolerant species (halophytes) constitutively exhibit a low level of Na^+/H^+ antiport activity and respond to salt by increasing the synthesis of antiporters, while in more salt-sensitive species such as *Ho. vulgare* and *He. annuus* salt invokes the activation or modification of pre-existing proteins, but the capacity to pump Na^+ (V_{\max}) does not increase with increasing external salinity.

19.3.2 A UBIQUITOUS TRANSPORTER?

Whether or not the tonoplast Na^+/H^+ antiporter is a ubiquitous transporter found in all plants remains in question (Mennen *et al.*, 1990). Activity of the tonoplast Na^+/H^+ antiporter, measured as Na^+ -dependent H^+ efflux in tonoplast-enriched membrane vesicles, has been documented in a number of halophytes (leaves of *Atriplex gmelini* (Matoh *et al.*, 1989) and *M. crystallinum* (Barkla *et al.*, 1995), and the roots of *At. nummularia* (Hassidim, *et al.*, 1990)), as well as in a number of salt tolerant glycophytes (*B. vulgaris* storage tissue and cells (Blumwald and Poole, 1985), *Ho. vulgare* roots (Garbarino and DuPont, 1988, 1989, DuPont 1992, Fan *et al.*, 1989), *G. hirsutum* (Hassidim, *et al.*, 1990), *Plantago maritima* (Staal *et al.*, 1991), *He. annuus* roots (Ballesteros *et al.*, 1997) and *Catharanthus roseus* cells (Guern *et al.*, 1989)). No evidence of tonoplast Na^+/H^+ antiport activity was found in either *L. esculentum* or *L. cheesmannii* (with or without exposure of the plants to salt), despite observations of plasma membrane Na^+/H^+ antiport activity (Wilson and Shannon, 1995). Mennen *et al.* (1990) attempted to address the question of ubiquity by surveying a range of species, and looking for metabolic Na^+ uptake in the presence of K^+ as evidence of the tonoplast Na^+/H^+ antiporter. Based on this methodology, it was concluded that the tonoplast Na^+/H^+ antiporter did not exist in *B. vulgaris*, *Ho. vulgare*, *L. esculentum* and *Triticum aestivum*, but was present in *G. hirsutum*, *Phaseolus vulgaris*, *Ricinus communis*, *Vigna radiata* and *Z. mays* (Mennen *et al.*, 1990). Because these determinations were made 1) only in roots, 2) in the absence of exposure of the plants to salt (with the exception of *G.*

hirsutum) and 3) using an indirect method of evaluation (rather than measurement of Na^+ -dependent H^+ -efflux from tonoplast membrane vesicles), the conclusion that the Na^+/H^+ antiporter is not ubiquitous should be regarded cautiously until more definitive assessments can be made.

19.3.3 CLONING OF Na^+/H^+ ANTIPORTER GENES.

The primary impediment to an accurate assessments of the existence and prevalence of the Na^+/H^+ antiporters in plants and a more detailed elucidation regarding its structure and function has been until very recently the inability to clone the genes encoding Na^+/H^+ antiporters from plants. The isolation of genes for Na^+/H^+ antiporters in bacterial (Pinner *et al.*, 1992, Padan and Schuldiner, 1994) and mammalian systems (Orlowski and Grinstein, 1997, Wakabayashi, *et al.*, 1997) has lagged behind the cloning of genes for other transporters such as the V-ATPase. Unlike the V-ATPase genes, there is only moderate sequence conservation amongst Na^+/H^+ antiporter genes from different organisms. This in large part accounts for the tremendous discrepancy between our knowledge pertaining to the regulation and expression of the V-ATPase under salinity, and that of the Na^+/H^+ antiporter.

The use of yeast as a model system to identify genes involved in salt tolerance in plants, the genome sequencing projects in *Saccharomyces cerevisiae* and *Ar. thaliana*, and the evidence for homologs in yeast of prokaryotic and eukaryotic Na^+ transporters has led to a breakthrough in the efforts to isolate Na^+/H^+ antiporter genes in plants. From *Sa. cerevisiae*, a homologue of the mammalian amiloride sensitive Na^+/H^+ antiporter was identified from the genome sequence based on sequence similarity to other known Na^+/H^+ antiporters (Nass *et al.*, 1997). Targeted disruptions of this gene (NHX1) led to a decrease in Na^+ tolerance at low pH (4-5) and supported a role for this gene in the mediation of salt tolerance in *Sa. cerevisiae*. NHX1 also appeared to be required for the sequestration of Na^+ into an intercellular pool in *Sa. cerevisiae*, presumed to be the vacuole (Nass *et al.*, 1997).

A plant homologue of NHX1 (*At*NHX1) has recently been cloned from *Ar. thaliana* (Apse *et al.*, 1999, Gaxiola *et al.*, 1999). When *At*NHX1 is overexpressed in yeast, the protein is localized in the prevacuolar compartment, and the NaCl sensitivity of the *nhx1* mutant is suppressed under certain conditions (Gaxiola *et al.*, 1999). When *At*NHX1 is overexpressed in *Ar. thaliana*, the protein is found predominantly in tonoplast and Golgi apparatus/endoplasmic reticulum enriched fractions. Vacuoles isolated from these transgenic plants exhibited higher Na^+/H^+ exchange rates compared to vacuoles from control plants, and this flux was both selective for Na^+ and electroneutral, supporting the function of the *At*NHX1 protein as a Na^+/H^+ antiporter (Apse *et al.*, 1999). Most significantly, overexpression of the *At*NHX1 gene conferred increased salt tolerance to transgenic *Ar. thaliana* plants (Apse *et al.*, 1999). The identification of Na^+/H^+ antiport genes in plants and the resulting availability of molecular probes and experimental approaches should greatly facilitate the elucidation of many questions regarding the role of the tonoplast Na^+/H^+ antiporter in the response of plants to salinity, including the issue of ubiquity, temporal and spatial patterns of induction, modes of regulations, structure, function, etc.

19.4 Membrane properties and passive transport: lipids, ion channels and aquaporins

19.4.1 POSSIBLE ROLES OF CHANGES IN TONOPLAST LIPID COMPOSITION FOR SOLUTE TRANSPORT AND STORAGE

Salinity-induced changes of the tonoplast lipid composition may influence the properties of the vacuolar membrane in two different ways: on one hand, they might modify passive membrane properties leading to a variation of the resistance for remobilization of ions sequestered in the vacuole; on the other hand they might influence the activity of tonoplast-intrinsic transport proteins due to their modification of the molecular environment.

From studies on pure lipid planar membranes and liposomes there is evidence that the lipid composition of the bilayer influences its permeability to water (for review, see Fettiplace and Haydon, 1980) and to anions, cations and non-electrolytes (Blok *et al.*, 1975, 1976). Major factors affecting the permeability of bilayers to ions (Li^+ , Na^+ , K^+ , Rb^+ , F^- , Cl^- , Br^- , SO_4^{2-}) were the fatty acid chain length and differences in the phase transition temperature of various lipid mixtures (Blok *et al.*, 1975). Thus, the ability to store salt in the vacuole is dependent upon not only the function of primary-active proton pumps and ion transporters in the tonoplast, but also an appropriate lipid composition of the tonoplast which facilitates the retention of ions in the vacuole as well.

Moreover, the membrane lipid composition might regulate the activity of transporters. A lipid dependency of enzyme activity has been demonstrated for the *V. radiata* V-ATPase (Yamanishi and Kasamo, 1993). A lipid modulation of activity might also be true for other tonoplastic transport proteins and thus specific lipid molecules might play an important role in the regulation of vacuolar salt accumulation if the tonoplast lipid composition is altered when plants are grown under saline conditions.

19.4.2 SALINITY-INDUCED CHANGES OF TONOPLAST LIPID COMPOSITION

Inspection of the tonoplast lipid composition in the halophyte *Su. maritima* (Leach *et al.*, 1990 b) revealed that the membrane seems to be optimized for minimization of passive ion permeability; the fatty acid chains are highly saturated, the membranes contain large amounts of sterols (cholesterol makes up 30% of the sterol fraction) and the phospholipid : protein ratio is high, ranging around a value of 1. The properties mentioned lead to a low membrane fluidity and, thus could be involved in prevention of passive ion fluxes from the vacuole to the cytoplasm. Sterols have been demonstrated to reduce membrane permeability for small molecules (Grunwald, 1968) and an increase in membrane free sterol content might be a general effect of salt treatment as could be shown by lipid analyses of total lipid extracts (Douglas and Sykes, 1985) and of plasma-membrane fractions (Douglas, 1985) from salt-treated *Citrus* roots (see also Kuiper, 1985). A high phospholipid : protein ratio could be interpreted to minimize destabilizing effects of protein insertion into the lipid bilayer. In this respect, it is interesting that Leach *et al.* (1990 b) found a large amount of glycolipids in the *Su. maritima* tonoplast.

It was suggested that these glycolipids stabilize lipid-protein interactions, therefore they may also contribute to a higher membrane rigidity.

Comparison of tonoplast vesicles isolated from well-watered and salt-treated plants of *M. crystallinum* after mechanical blending of the tissue revealed no changes in the overall lipid composition with the exception that the level of free sterols and the proportion of saturated fatty acid chains was higher in preparations from salt-treated plants (Haschke *et al.*, 1990). Kliemchen *et al.* (1993) could demonstrate by electron-paramagnetic resonance studies that salt-treatment led to a decrease in the fluidity of the *M. crystallinum* tonoplast. Since the protein composition of the *M. crystallinum* tonoplast drastically changes during salt-treatment (Bremberger and Lüttge, 1992, Ratajczak *et al.*, 1994) the decrease in tonoplast fluidity might not only be due to the lipid changes mentioned above. Changes in tonoplast protein content and protein composition can alter tonoplast fluidity, as was demonstrated for phenotypic adaptations of the *K. daigremontiana* tonoplast fluidity in response to plant cultivation at different temperatures (Behzadipour *et al.*, 1998).

Although studies on salt-effects on tonoplast lipid composition obtained by inspection of purified tonoplast vesicles are rare, one can conclude from the findings mentioned above and by taking into account data obtained with crude lipid extracts from salt-treated salt-tolerant and salt-sensitive plants (Blits *et al.*, 1990, Zenoff *et al.*, 1994) that a general effect of salinity is a decrease in membrane fluidity leading to a decrease in passive ion fluxes across the membrane. The decrease in membrane fluidity seems to be mainly due to an increase in the free sterol content and a higher proportion of unsaturated fatty acid chains.

19.4.3 POSSIBLE ROLE OF ION CHANNELS IN SALINITY RESPONSES

Advances in patch clamping techniques have enabled researchers to observe and record passive fluxes across plant membranes, and to begin characterizing the nature and identity of the mechanisms responsible for these fluxes (Hedrich and Schroeder, 1989). Ion channels, which facilitate the flux of ions across the membrane through intrinsic membrane proteins, typically mediate a considerably more rapid flux of ions than occurs via carriers. While the majority of studies have focused on fluxes across the plasma membrane, details are emerging regarding ion channels in the tonoplast, and how they might contribute to ion homeostasis under conditions of elevated salinity (for review, see Tyerman and Skerrett, 1999).

Despite the increasing number of reports describing various ion channels in the tonoplast, relatively little is understood yet about their role in the response of plants to salinity. It has been suggested that the role of ion channels during the response of a cell to hypersalinity could include one or more of the following: 1) osmotic or net flux, 2) gradient control, 3) signaling (Tyerman and Skerrett, 1999). And it can be readily imagined that the physiological perturbations associated with salinity, i.e. changes in cytoplasmic pH, calcium concentrations, membrane pds, mechanical stress (changes in turgor, osmotic swelling/shrinkage) could alter the behavior and activity of ion channels, since the activity and gating of many of the tonoplast ion channels have been shown to be sensitive to these factors. The control of passive fluxes, especially of Na^+ , has long been proposed to be of importance to salt tolerance. In order for Na^+ to be

sequestered in the vacuole, there would need to be restricted passive flux of Na^+ back into the cytosol. Otherwise, steep concentration gradients between cytoplasm and vacuole could not be achieved, or would be energetically very expensive to sustain. Data from two studies support this concept. Pantoja *et al.* (1989), in a comparison of tonoplast ion channels from *B. vulgaris* (halophyte) and *L. esculentum* (glycophyte), observed a low channel conductance in *B. vulgaris* under physiological pds, relative to *L. esculentum*. This was interpreted as evidence for the need of the halophyte to maintain Na^+ gradients and restrict Na^+ flux across the tonoplast. When *Pl. maritima* plants were grown in salt, there was a dramatic decrease in the probability of channel opening, again suggesting a role for reduced channel activity to support Na^+ sequestration in the vacuole (Maathuis and Prins, 1990).

19.4.4 SALINITY RESPONSE OF AQUAPORINS

It has been almost 10 years since the existence of membrane integral proteins in plants, exhibiting high sequence homology to members of the major intrinsic protein (MIP) family (Guerrero *et al.*, 1990, Johnson *et al.*, 1990, Höfte *et al.*, 1992, Yamaguchi-Shiozaki *et al.*, 1992) were first reported. Some of these MIPs are present at the plasma membrane (plasma membrane intrinsic proteins, PIPs; Chapter 18), while others are located on the tonoplast (tonoplast intrinsic proteins, TIPs). MIPs initially were found to be present in large amounts in bovine eye lens fiber membranes (Gorin *et al.*, 1984). They exhibit apparent molecular masses ranging from 20 to 30 kDa, contain 6 membrane-spanning domains and tend to form dimers or tetramers (for reviews, see Verkman, 1993, Chrispeels and Agre, 1994). MIPs have been shown to transport glycerol, urea, ions and water through biological membranes (for review, see Agre *et al.*, 1995). Water transporting MIPs, which are denominated aquaporins, might play a role in plant's response to salinity since changes in MIP expression could regulate water permeability of both the plasma membrane and the tonoplast. The first evidence for the presence of aquaporin activity in the tonoplast of plant cells came from Maurel *et al.* (1993) who expressed an *Ar. thaliana* γ -TIP in *Xenopus* oocytes and demonstrated γ -TIP-dependent facilitated osmotically driven penetration of water molecules through the oocyte membrane. Meanwhile aquaporin function has been characterized for several other plant TIPs and PIPs (for reviews, see Maurel, 1997, Schäffner, 1998).

By screening *M. crystallinum* root and leaf cDNA libraries Yamada *et al.* (1995) isolated 5 transcripts exhibiting sequence homology to PIPs which were expressed in a tissue-specific manner. PIP transcript amount decreased transiently to different extents after salt stress and recovered again within one or two days. Ongoing work in the laboratory of Hans J. Bohnert revealed that there are at least 20 different MIP-like proteins (including PIPs and TIPs) in *M. crystallinum*, some of which decline following salt stress, while others do not. Moreover, some MIPs seem to decline under drought conditions more than under saline conditions (H.J. Bohnert, personal communication). In suspension cultured tobacco cells, TIP gene expression appears to decline in response to NaCl, and in cells adapted to NaCl there is a decreased permeability of the tonoplast to water (C. Maurel, personal communication). Probing of tonoplast-enriched membrane fractions isolated from *M. crystallinum* leaves with a polyclonal antiserum against the radish γ -TIP (Maeshima, 1992) led to immunodecoration of a 23 kDa polypeptide on Western blots. The abundance of this protein relative to total tonoplast

protein decreased with increasing sap salt concentrations in plants irrigated with 400 mM NaCl for 12 days (M. Maeshima and R. Ratajczak, unpublished). Taken together, there are hints that there is a short-term or long-term decrease in tonoplast aquaporin content under saline conditions in *M. crystallinum* which could decrease the rate of water exchange between the cytoplasm and the vacuolar lumen.

19.5 Conclusions and future perspectives

It becomes apparent when reviewing the literature that it is still difficult to draw general conclusions about the role and the responses of tonoplast proteins to salinity. Even for the intensively studied V-ATPase the results at the protein level are contradictory, and one of the only conclusions that can be drawn is that the V-ATPase protein amount is up-regulated due to salinity, at least in most halophytes and salt-tolerant species. Reports about changes in V-ATPase hydrolytic activity have to be discussed cautiously since in most cases activities related to total tonoplast protein have been determined instead of specific activities related to V-ATPase protein. However, looking at the data available it could be suggested that salinity does not affect specific ATP-hydrolysis activity of the V-ATPase and that an increase in ATP-hydrolysis activity related to tonoplast protein is due to an increase in V-ATPase protein. An unresolved question is whether salinity could lead to a modification of the coupling ratio of proton translocation and ATP hydrolysis. Efforts should be made to answer this question, since this would provide the opportunity to learn more about the molecular mechanism of ATP-driven proton translocation of the enzyme. In general, transcript levels of V-ATPase subunits seem to be up-regulated after salt-treatment, however in most studies it was not determined whether changes in transcript levels were correlated with increased abundance of V-ATPase protein.

Even less data are available regarding the responses of other tonoplast proteins to salinity, e.g. the V-PPase, the Na^+/H^+ antiporter and ion channels. Although sequence information is available for a large number of V-PPases from different species data on salt-induced changes have been reported at the protein level only. While plant genes for putative Na^+/H^+ antiporters have recently been cloned providing the possibility to study salt-effects on the their expression, sequence information about ion channels is still lacking. This of course is mainly due to the low abundance of these proteins, but hopefully data from genome sequencing projects will lead to the molecular identification of these and other transporters. Relatively little attention has been paid to the investigation of the role of the tonoplast lipid environment in salinity response, although changes in the rigidity of the tonoplast might have a considerable impact on vacuolar salt storage. Studies on promoters of genes coding for tonoplast proteins involved in salinity response are in the early stages and the involvement of different signal transduction pathways has yet to be resolved. However as genes for more transporters are sequenced and compared, clues regarding mechanisms of regulation and signal cascades should emerge.

There clearly is a need to study salinity responses of tonoplast proteins in more detail and in a more coordinate manner, so that conclusions can be drawn from a composite of data from the protein level, the gene expression level and the level of regulation of gene

expression. It will be important that future examinations of the function of tonoplast transporters recognize the unique role that different cells, tissues and organs are likely to play in the response of plants to salinity. It is highly likely that different roles and degrees of importance will be assessed to different transporters in different cells. Advances in *in situ* methodology, advanced imaging and microanalytical procedures should facilitate the more focused examination of transporters within a given cell. Extending the studies of tonoplast transporters to include comparisons between species exhibiting different levels of salt-tolerance will further assist in defining which transporters and which mechanisms are pivotal to ion homeostasis and the ability of plants to tolerate salinity.

19.6 References

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CHAPTER 20

GENETICS OF SALINITY RESPONSES AND PLANT BREEDING

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Abstract

Molecular biology offers the possibility of designing new plant genotypes with enhanced resistance to salinity. This does not, however, mean that understanding the genetics and physiology of responses to salinity is complete or no longer necessary. To be able to exploit the new technologies it is important to understand the inheritance of traits and how they can be integrated in complex, highly structured whole plants through varying phases of development. In this chapter we consider the constraints and possibilities of breeding crops for salt resistance from the viewpoint of the different disciplines of agronomy, breeding, physiology and molecular biology.

20.1 General introduction

Although tolerance to salinity is found in a wide range of plant taxa, this chapter necessarily concentrates on agriculturally important crops, and on model systems for molecular studies (rice and *Arabidopsis*). The ecological and population genetics of halophytes have received relatively little attention. In addition to the other chapters in this volume, the reader is directed to two recent volumes that include chapters on the general understanding and genetics of plant responses to salinity (Jaiwal *et al.*, 1997; Yeo and Flowers, 1994). General reviews that illustrate the complexity of salt tolerance, written from different perspectives, include those of Blum, (1988), Lerner *et al.* (1994), Munns (1993), and Neumann (1997). Other authors have given specific consideration to breeding strategies (Ashraf, 1994; Flowers and Yeo, 1995; Tal, 1985), ion transport mechanisms (Maathuis and Amtmann, 1999; Niu *et al.*, 1995; Schachtman and Liu, 1999) and molecular studies (Flowers *et al.*, 1997; Grover *et al.*, 1993, 1998, 1999; Jain *et al.*, 1997; Yeo, 1998).

In this chapter we have assumed that the reader has a basic understanding of the responses of plants to salt stress, including the nature of the stresses associated with salinity (osmotic, toxic ions and nutritional) and the time scale of different responses (Munns, 1993), the theory of solute compartmentation within cells (Chapter 8) and compatible solutes (Chapter 9), and the differences between cellular and whole plant responses (Chapter 8).

A question that is often asked is 'Why have so few salt-tolerant varieties been developed after decades of extensive study of salinity in plants?' (e.g. Flowers and Yeo, 1995, 1997; Munns, 1993) One answer is that reports of development and selection of salt-tolerant varieties are not always easily accessible. In general, large seed companies and international research centers have other priorities, and much of the selection work is done in national breeding programs, primarily for local consumption. Successes are often only reported, if at all, in Institutional Annual Reports, Conference Proceedings etc. (Table 1). Uptake by farmers often depends on other desirable characteristics (high yield potential, market acceptance, appropriate phenology, disease resistance *etc.*) as much, or more than, on salt tolerance *per se*.

Three particular factors make selection in the field difficult. The first relates to the nature of the stress or stresses. Salinity in the field is not a simple stress, but may be caused by excessive amounts of any combination of a range of cations (Na^+ , Ca^{2+} , Mg^{2+}) and anions (Cl^- , SO_4^{2-} , HCO_3^-). In sodic soils excess Na^+ dominates the cation exchange sites on soil particles, and may be present in the alkaline soil solution in relatively low concentrations associated with carbonate or bicarbonate anions (see Chapter 2). In addition, salinity may be accompanied by other adverse environmental factors such as poor drainage, impenetrable soil, water deficit and excessive heat or cold. Thus one site cannot act as a model for all saline fields.

The second problem relates to the spatial and temporal variation in salinity within a given field (see below), making accurate selection of varieties a statistical problem, and selection from segregating populations a lottery. A third consideration is the multigenic nature of salt tolerance, bringing into play the law of limiting factors. The performance of the organism as a whole (in this case its salt tolerance) is determined by the factor that is most limiting (for example, the ability to exclude Na^+ from the shoot, or to partition ions between the symplast and apoplast - see Chapters 6, 8, 18 and 19). The most salt-tolerant genotype will be one in which all mechanisms are optimized, and working in concert under specific environmental conditions. The chances of producing such a genotype by the random combination of parental genes, and of being able to recognize a unique individual in an heterogeneous saline field, are quite small.

In the rest of this chapter the idea of breeding for salt tolerance is examined from the viewpoint of an agronomist, a breeder, a physiologist and a molecular biologist, with the intention of demonstrating that a multi-disciplinary collaboration is likely to be ultimately more successful than a specialist understanding of part of the problem.

TABLE 1. Salt- and sodicity-resistant genotypes. This list includes both existing genotypes selected for resistance and new varieties developed specifically for resistance, and is illustrative rather than comprehensive.

Species	Genotype	Reference
Rice	CSR1, CSR4, CSR10, CSR13, CSR21, CSR27, CSR29 <i>etc.</i>	Sinha and Bandyopadhyay, 1984; Flowers and Yeo, 1995; Anon., 1996, 1997; Mishra <i>et al.</i> , 1996
	RST-24	Sajjad, 1990
	At 69-2	Chaubey and Senadhira, 1994
	PVR-1, MCM-1, SR 1022, SR 1-2-1, MR-18, Co 43, Usar 1	Rana, 1986
	MK 47-22, SR 3-9, Panvel 28-23, Panvel 11-2, Panvel 5-30, Panvel 32-10-1-1	Salvi and Chavan, 1983
	Yeonggwang, Gancheok 9, Namyangbyeon, Seohaebyeon, Janganbyeon, Seonbyeon, Gyeonhwaebyeon, Yeongdeogyeon, Donghaebyeon	Chaubey and Senadhira, 1994
	Jhona 349	Akbar and Yabuno, 1975, 1977; Akbar <i>et al.</i> , 1972
	Aiwu	Lutts and Guerrier, 1995
	80-85	Wang <i>et al.</i> , 1988
	Pokkali, Jhona 349, Nona Bokra, Kalarata, Damodar, PSBRc 48 Bicol, PSBRc 50 Hagonoy	Akbar <i>et al.</i> , 1986a,b
Sorghum	Double TX	Francois <i>et al.</i> , 1984
Wheat*	Kharchia 65, KRL 1-4, KRL 3-4, KRL 13, KRL 19, KRL 20, KRL 22	Anon., 1996, 1997
	Lu 26s	Qureshi <i>et al.</i> , 1980
	PI 178704, PI 178012, PI 180988	Kingsbury and Epstein, 1984
	SARC-1, SARC-3	Ashraf and O'Leary 1996a,b
	S24	Ashraf and Khanum, 1997
	HD 2560	Minhas, 1998
Alfalfa	Raj 3077	Bhatnagar and Sharma, 1996
	AZ-GERM SALT-I	Dobrenz <i>et al.</i> , 1983
	AZ-GERM SALT-II	Dobrenz <i>et al.</i> , 1989
	AZ-9ONDC-ST	Johnson <i>et al.</i> , 1991
	CUF 101-T ₂	Noble <i>et al.</i> , 1984
cordgrass	Euver S ₁	Ashraf <i>et al.</i> , 1987
cordgrass	Avalon	Hamer <i>et al.</i> , 1988
Maize	Arizona 8601	Day, 1987
cotton	NIAB 78	Anon., 1982
	MESR 16	Minhas, 1998
Mustard	CSTR 330-1	Anon., 1996
	DIRA 342	Minhas, 1998
Chickpea	CSG 8962	Anon., 1997

* see <http://www.bangor.ac.uk/~azs809/salinity/Resources/Germplasm.htm>

20.2 An agricultural perspective

For the ecologist, competition for resources is a major factor determining plant distribution and reproductive success. In extreme environments such as saline deserts this may reduce to the simple ability to survive. For the agronomist, however, the goal is usable biomass or yield. In very saline conditions this may mean growing a halophytic forage rather than trying to grow a conventional grain crop. One argument for trying to improve the tolerance of glycophytic crops to salinity and sodicity is that it could increase the lifetime of irrigation systems that use low quality water or are subject to rising water tables. Historically most irrigated systems in semi-arid areas have succumbed eventually to secondary salination. The current state of the problem has been summarized for a number of countries by Ghassemi *et al.* (1995; see also Chapter 1). Difficulties often arise because of inadequate leaching and drainage. Installing adequate drainage systems in new or old irrigation schemes is very expensive and difficult to justify on short-term economic grounds or where the value of the crop is low, but in the long term is the most sustainable solution if the supply of water is sufficient.

It has been suggested (Tanton, 1991) that breeding enhanced salt resistance into crop plants may be counter-productive as it may decrease the pressure to ensure appropriate water management and could in time contribute to degradation of agricultural systems by increasing salinity and waterlogging. It is clear that enhanced salt tolerance can only be of medium to long term value under prescribed circumstances. A long term salt and water balance must be maintained in the soil regardless of the salt tolerance of the crop being grown. Enhanced tolerance, however, can increase the acceptable equilibrium soil salinity. In some cases such as the fodder species *Leptochloa fusca*, a deep rooting salinity- and sodicity-tolerant species, a crop may also improve soil drainage and help to reverse the deterioration in soil salinity and water balance. One condition in which enhanced crop tolerance is clearly advantageous is where brackish water is available for irrigation of sandy, free-draining soils.

The problem of heterogeneity of field salinity (Castrignano *et al.*, 1994; Hajrasuliha *et al.*, 1980; Lesch *et al.*, 1995; Rhoades *et al.*, 1997; Richards and Dennett, 1980; Shainberg and Shalhevet, 1984) has been eluded to earlier. From an agricultural perspective the patchiness, at whatever scale from field to country, leads to the question of the extent to which the saline areas contribute to overall yield. Richards (1983, 1992, 1995) has advocated selection for high yield under low salinity (high yield potential), since, in some agricultural systems, most of the yield comes from the areas of low salinity. Testing at low, intermediate and high salinity was preferred by Iguartu (1995). The choice of selection environment might depend on the extent and variability of salinity in the target environment. Where saline areas are a small proportion of the total area, breeding for yield potential is an attractive strategy. If salinity is more widespread there is a case for breeding for salt tolerance, at least for the saline areas. Part of the uncertainty comes from genotype x environment interactions, where the ranking of varieties for yield changes as the stress increases. Associated with this is the

idea that there is necessarily a yield penalty associated with tolerance to salinity - tolerance being linked with low yield potential.

Breeding for salt tolerance cannot be an end in itself. A new variety must be competent in the target environment (*i.e.* locally adapted in terms of phenology, disease resistance etc.) and acceptable to the farmer and the end-users, be they subsistence farming communities or industrial companies. It must also have clearly demonstrable advantages over existing varieties to gain wide acceptance.

20.3 A plant breeders perspective

Conventional breeding for salt tolerance has three prerequisites - sufficient variation for the trait being selected, high heritability of the trait and selection procedures applicable to large populations. Variation may be pre-existing or induced by mutation or tissue/cell culture.

20.3.1 VARIATION WITHIN ACCESSIONS

Variation within an existing variety may exist where the variety has not been subjected to selection pressure for salt-related traits such as Na^+ accumulation. Few varieties, particularly traditional varieties and landraces, are completely homozygous. Intra-varietal selection has been reported in alfalfa (Noble *et al.*, 1984; Allen *et al.*, 1985; Al-Khatib *et al.*, 1993), maize (Ashraf and McNeilly, 1990), wheat (Rashid *et al.*, 1999), rice (Yadav *et al.*, 1996; Yeo, 1992; Yeo *et al.*, 1988) and other crops (Ashraf *et al.*, 1987). In wild species relatively salt-resistant ecotypes have been identified in several species (Ashraf *et al.*, 1986a,b, 1987).

20.3.2 VARIATION BETWEEN ACCESSIONS

Extensive germplasm collections exist for the major crop species, and databases, catalogues and descriptor lists are usually readily available. It is more difficult to find detailed information about salinity tolerance or the salinity of the site at which the collection was made - data that are particularly useful for landraces and wild relatives. Given the problems of field heterogeneity mentioned above, screening very large collections adequately for salt tolerance in the field has rarely been attempted. Often a subset of genotypes is screened in one year, and promising lines re-examined in subsequent years. Alternatively, testing is done at the germination and/or seedling stage in the more controlled conditions of a growth chamber or greenhouse (*e.g.* Mano *et al.*, 1996 for 6712 barley genotypes and 368 isogenic lines). A few examples of accessions selected from inter-varietal screens are given in Table 1. The problem of deciding on selection techniques and criteria is addressed later in this chapter.

20.3.2.1 Soybean

In soybean the difference between the salt-sensitive cv. Jackson and the salt-resistant cv. Lee was largely attributed to a single gene (*Ncl*) that restricted chloride accumulation in Lee (Abel, 1969; Abel and McKenzie, 1964). From physiological studies on the same cultivars, it was suggested that the leaf necrosis observed in Jackson after salt treatment was caused by increasing accumulation of Na^+ in the leaves and the depression of Ca^{++} uptake and translocation, in addition to insufficient control of Cl^- transport in cv. Jackson (Läuchli and Wieneke, 1979; Wieneke and Läuchli, 1979, 1980). Studies by Shao *et al.* (1994) of segregation ratios for salt resistance in F_1 , F_2 , F_3 and backcross generations in crosses between salt-sensitive and salt-resistant soybean cultivars confirmed that one pair of genes was responsible for resistance, with resistance being dominant. Zhong *et al.* (1997) identified three molecular markers that were present in salt-resistant cultivars (Morgan and Wenfeng 7) but not in the sensitive cultivars Hark and Jackson. Differences in Cl^- accumulation are also responsible for relative salt resistance in *Citrus* (Sykes, 1985) and *Vitis* (Sykes, 1992).

20.3.2.2 Rice

Extensive physiological and genetic studies of relative salt resistance in rice accessions at the University of Sussex (UK) have provided ample evidence that a variety of different mechanisms contribute to resistance. There is a clear link between the accumulation of Na^+ in rice leaves and salt-induced injury. Salt resistant accessions such as the tall *indica* landraces Nona Bokra, Pokkali and the cultivar CSR1 generally have lower leaf Na^+ concentrations than sensitive ones such as I Kong Pao, IR 26 and IR 28 (Akbar, 1986; Akbar *et al.*, 1986a,b; Lutts and Guerrier, 1995; Lutts *et al.*, 1995; Welfare *et al.*, 1996; Won *et al.*, 1992; Yeo and Flowers, 1983). Some of this variation for Na^+ accumulation within and between accessions can be ascribed to differences in rates of apoplastic transport across the root, also called bypass flow (Yadav *et al.*, 1996; Yeo *et al.*, 1987), and to differences in transpiration rates and water use efficiency (Flowers *et al.*, 1988). Other factors such as effective compartmentation of Na^+ and the accumulation of Cl^- are, however, also important (Flowers *et al.*, 1985, 1991; Gorham and Akhtar, unpublished). As can be seen from Table 1 there are a large number of rice genotypes that have been selected for salt resistance, and the literature contains many other examples. A search of the IRRI rice bibliography (<http://ricelib.irri.cgiar.org/>) produced 385 references to [salt tolerance rice], and over 30 to [salt tolerance rice breeding/selection].

20.3.2.3 Wheat

There are a large number of references describing variation for salt resistance in wheat. As examples we can cite Kingsbury and Epstein (1984) who screened several thousand accessions and identified a few that were able to survive and produce grain at high salinity, Qureshi *et al.* (1980) who examined 12 spring wheat cultivars in Pakistan, and the Central Soil Salinity Research Institute in Karnal, India, where several salt- and sodicity-resistant lines have been identified (Anon., 1996, 1997). These examples illustrate the use of different screening techniques in terms of the substrate employed,

but are all based on biomass or yield. Other workers have made selections based on Na^+ accumulation or uptake (Nevo *et al.*, 1992), or have explored links between phenology and salt resistance (Taeb *et al.*, 1992). As in rice, there appears to be a wealth of variation between accessions that could be exploited in breeding for salt resistance.

20.3.2.4 Barley

Mano *et al.* (1996) found variation for salt resistance in a screen of over 6,000 barley genotypes. Varieties from China and Korea were more resistant than varieties from Turkey and Japan. Six-rowed, naked, Oriental types with a non-brittle rachis and non-uzu types were more tolerant than two-rowed, covered, Occidental types and uzu or semi-brachytic types. A similar difference was found for isogenic lines, indicating that *v*, *n* and *uz* genes affected salt tolerance. Gorham *et al.* (1994 and unpublished) also found considerable variation in barley accessions, but the ranking depended on how the salt was applied. Some accessions were more tolerant of soil salinity, but less tolerant of foliar salt application.

20.3.3 VARIATION BETWEEN SPECIES

There are many examples of wild relatives of crop species within the same genus that are more salt-resistant than the cultivated forms. There are taxonomic uncertainties about the limits of genera that confuse the distinction between variation within species and with more distant taxa. Examples are the inclusion of *Aegilops* within *Triticum* (Bowden, 1959) and the separation of many *Hordeum* species into *Critesion* (Löve, 1982).

20.3.3.1 Barley

The closest relative of cultivated barley is the wild species *Hordeum spontaneum*. Variation for salt-resistance in this species has been studied extensively by Nevo and co-workers (Forster *et al.*, 1997; Nevo *et al.*, 1993; Handley *et al.*, 1997a,b) and is being exploited for the development of molecular markers (Ellis *et al.*, 2000; Forster *et al.*, 2000; Pakniyat *et al.*, 1997). Some of the more distantly related wild barley species, such as *H. jubatum* (Huang and Redmann, 1995a,b,c; Huang *et al.*, 1996; Suhayda *et al.*, 1992) and *H. chilense* (Martin *et al.*, 1998) are even more resistant to salinity. The wild species also show considerable variation for K^+/Na^+ discrimination (Gorham, 1992, 1993). A fundamental problem here is the breeding barriers that exist between the cultivated and wild species (Von Bothmer, 1992), although work with *H. chilense* hints at the possibilities (Forster *et al.*, 1990). Similar problems exist with the wild relatives of rice (see below) and soybean (Singh *et al.*, 1998).

20.3.3.2 Lycopersicon

A number of wild relatives of tomato are known to be relatively salt-resistant (Tal and Katz, 1980; Taleisnik-Gertel *et al.*, 1983). One of the earliest attempts at interspecific hybridization to improve salt resistance was that of Lyon (1941). *Lycopersicon pimpinellifolium* was more resistant to Na_2SO_4 salinity than the cultivated tomato, *L.*

esculentum. Interspecific hybrids were, however, more like the sensitive parent. A more recent study (Asins *et al.*, 1993) has revealed some of the complexity of inheritance of salt-resistance-related traits in a cross between these two species. Selections from an F_2 population of a cross between *L. esculentum* and *L. cheesmanii* were more resistant to NaCl than the cultivated parent (Rush and Epstein, 1976). A study of six *Lycopersicon* species revealed that variation for salt resistance was paralleled by resistance to cold temperatures (Foolad and Lin, 1999). Molecular markers and QTL for salt resistance will be discussed in section 20.5.2 below.

20.3.3.3 *Solanum*

Plants of the wild species *S. kurzianum* were found to be more salt tolerant than those of the cultivated species *Solanum tuberosum* cvs Alpha and Russet Burbank by Sabbah and Tal (1995). Growth was less affected by salinity in the wild species, but accumulation of sodium in the shoots was greater, and accumulation of potassium less than in *S. tuberosum*. Callus derived from the wild species was not more tolerant than that from the cultivated species. *S. kurzianum* was suggested as a potential source of genes for increasing the salt tolerance of potato based on the similarity of the physiological responses to salt stress between *S. kurzianum* and the wild salt-tolerant relatives of tomato.

20.3.3.4 *Wheat*

Differences in K^+ and Na^+ accumulation were observed in shoots of different wheat and *Aegilops* species at low ($<100 \text{ mol m}^{-3} \text{ NaCl}$) salinities in hydroponic culture (Wyn Jones, Gorham & McDonnell, 1984; Shah *et al.*, 1987). Greater discrimination (in favour of K^+ and against Na^+ accumulation) was shown by hexaploid bread wheat (*Triticum aestivum* L.) than by tetraploid durum wheat (*T. turgidum* L.). *Aegilops tauschii* Cosson. (syn. *Ae. squarrosa*), the source of the D genome in bread wheat, also exhibited high discrimination between K^+ and Na^+ , and it was concluded that the D genome was responsible for the difference between tetraploid and hexaploid wheat. Other *Aegilops* species related to the B genome of wheat did not show enhanced K^+/Na^+ discrimination (Gorham, 1990b). Further investigations (Gorham, *et al.*, 1991; Gorham, 1993) suggested that although the trait was exhibited by diploid wheats (*T. monococcum* L. and *T. urartu* Thum.), and survived in the tetraploid species *T. timopheevi* Zhuk. (genome SSAA), it had been lost during the evolution of *T. turgidum* (genome BBAA). Within the tribe Triticeae the enhanced K^+/Na^+ discrimination trait was also observed in U-, M- and T-genome *Aegilops* species (Gorham, 1990b), rye and triticales (Gorham, 1990a) and synthetic hexaploid wheats derived from tetraploid wheat and *Aegilops tauschii* (Gorham 1990c). It was not exhibited by cultivated barley (*Hordeum vulgare* L.) or its wild relative *H. spontaneum* (Gorham *et al.*, 1990a), or by perennial wheatgrasses (Gorham, 1994), both of which are more salt-tolerant than bread wheat (King *et al.*, 1997). Evidence for the presence of enhanced K^+/Na^+ discrimination in other wild barley species is contradictory (Gorham, 1992, 1993).

Disomic substitution lines, in which D-genome chromosomes replaced their A and B genome equivalents in durum wheat cv. Langdon, were used to locate the trait on the

long arm of chromosome 4D (Gorham *et al.*, 1987). Further investigations with ditelosomic and nulli-tetra bread wheat lines indicated that the long arm of chromosome 4D was involved. Gene dosage had little effect on expression of the trait since lines tetrasomic for chromosome 4D had similar leaf K^+ and Na^+ concentrations to disomic 4D lines.

Since some aneuploid lines showed better relative salt tolerance than durum wheat, but (as aneuploids) had low yield potentials, Chinese Spring chromosome 4D from the Langdon disomic 4D(4B) substitution line was recombined with chromosome 4B in a tetraploid wheat background using the homoeologous pairing mutant *PH1c* in cv. Senator Cappelli (Dvorak & Gorham, 1992). Initially this produced 39 families of 4D/4B recombinant lines of which nine lines exhibited the enhanced K^+/Na^+ discrimination trait. Na^+ concentrations in young leaves fell into two non-overlapping classes.

In general, recombinant lines that exhibited the enhanced K^+/Na^+ trait were slightly more tolerant of salinity in the field than recombinants lacking the trait (Dvorak *et al.*, 1994). There was, however, considerable variation between individual lines, which is understandable considering that they originated from a hybrid involving two very different genetic backgrounds. Physiological investigations showed that *Kna1* affects transport of K^+ and Na^+ to the shoots, with little effect on root ion concentrations or on anion accumulation in leaves, and that the main site of action is probably at xylem loading in the roots (Gorham *et al.*, 1990b). Although the trait can be demonstrated at all salt concentrations, it is most apparent at low salinities ($<100 \text{ mol m}^{-3}$). At higher salinities other mechanisms which control ion accumulation appear to be more important (Gorham *et al.*, 1997).

20.3.3.5 Sunflower

Sunflower germplasm lines released in 1989 included salt-resistant interspecific crosses with *H. paradoxus* (Seiler, 1991). Miller (1995) examined the salinity resistance of five interspecific lines derived from crosses between *H. paradoxus*, a salt-resistant wild species of sunflower and the cultivated *H. annuus*. Three lines were more resistant than *H. annuus* to NaCl in both seedling and germination tests. These lines were crossed with a salt-susceptible inbred line. Segregation ratios of F2 populations and backcross progeny indicated that a major dominant gene, *Sal*, controlled seedling tolerance to salinity.

20.3.3.6 Rice

Oryza glaberrima, the African cultivated rice, is quite closely related to *O. sativa*. In tests at the seedling stage it was intermediate for survival between the salt-sensitive *O. sativa* cultivar IR28 and the salt-resistant cultivar Nona Bokra (Akbar *et al.*, 1987), but another report ranked the *O. glaberrima* accessions 102377 and 63-83 as more resistant than the salt-resistant *O. sativa* cultivar Pokkali (IRRI, 1978). Interspecific hybrids between these two species have been produced and advanced lines developed with the aid of backcrossing to *O. sativa* and doubled haploid techniques (Jones *et al.*,

1997a,b,c; WARDA, 1996,1997). Several other, more distantly related, *Oryza* species were more salt-susceptible than *O. glaberrima* (Akbar *et al.*, 1987).

20.3.4 WIDE HYBRIDIZATION

Porteresia (*Sclerophyllum*) *coarctatum* has been identified as a salt-resistant wild relative of rice (Bal and Dutt, 1986; Flowers *et al.*, 1990). As in many wild relatives there are barriers to direct hybridization between the wild and cultivated species. These may be overcome by embryo rescue into tissue culture or somatic hybridization (Akbar, 1986; Akbar *et al.*, 1986b; Brar *et al.*, 1997; Farooq *et al.*, 1992; Jelodar *et al.*, 1999; Jena, 1994, 1996).

Perennial wild species that are closely related to annual *Triticum* crops are more resistant to salinity than the crop species (Dewey, 1960; Gorham *et al.*, 1984, 1985; Hunt, 1965; McElgun and Lawrence, 1973; McGuire and Dvorak, 1981; Roundy, 1985a,b; Roundy *et al.*, 1985; Shannon, 1978). Two series of species formerly belonging to the genus *Agropyron* have received particular attention. These include the diploid *Thinopyrum bessarabicum* and its polyploid relatives *Th. junceiforme* and *Th. junceum*, and the diploid *Lophopyrum elongatum* and the polyploid *L. ponticum*. These species have been hybridized with wheat (Dvorak and Ross, 1986; Forster and Miller, 1985; Forster *et al.*, 1987; Gorham *et al.*, 1986; Mujeeb-Kazi *et al.*, 1993; Storey *et al.*, 1985) and alien chromosomes and chromosome regions that contribute to salt resistance have been identified (Deal *et al.*, 1999; Dvorak *et al.*, 1985; Forster *et al.*, 1988; Kasai *et al.*, 1998; King *et al.*, 1997; Koebner *et al.*, 1996; Omielan *et al.*, 1991; Zhong and Dvorak, 1995a,b). These studies, and those on the enhanced K^+/Na^+ gene *Kna1*, have confirmed that salt resistance is under multigenic control, with genes in chromosome groups 3, 4 and 5 having large effects.

Exploitation of wide hybrids may proceed along two lines. The chromosome number of the initial hybrid can be doubled with colchicine and the resulting amphidiploid used directly, or the desirable genes can be introduced into the background of the crop species. The former suffers from undesirable genes being expressed in the amphidiploid resulting in poor agronomic characters such as late flowering or difficulty in threshing. There is also a danger of gradual loss of the alien chromosomes. Given the number of genes involved, introgression of desirable genes is likely to be a lengthy process, but one that can be assisted by marker-assisted selection and pyramiding (see below).

20.3.5 INDUCED VARIATION

20.3.5.1 Mutations

Mutation is a useful tool in plant breeding and physiology. The *scabrous diminutive* mutant of pepper is a small, wilted pepper with poor K^+/Na^+ discrimination that is useful for studies of ion transport (Benzioni and Tal, 1978; Tal and Benzioni, 1977). In *Ceratopteris richardii* the salt-resistant mutants *stl1* and *stl2* have also proved valuable for physiological studies (Voglein *et al.*, 1996; Warne *et al.*, 1996, 1999). Variation for

salt tolerance has been induced in rice, with the mutant IR6-18 being more resistant than IR6 (Bari and Hamid, 1988). Chandhry *et al.* (1986) identified two M_3 lines that were more resistant than the parent variety Taichung 65. Mutants have also been useful in identifying salt-related molecular markers in rice (Zhang *et al.*, 1994, 1995a) and in studies of differential gene expression (Zhang and Chen, 1996; Zhang *et al.*, 1996, 1999). A reduction shoot Na^+ content was found in the barley cultivar Golden Promise, which is a mutant derived from the cultivar Maythorpe (Forster *et al.*, 1994). Kueh and Bright (1982) described mutants of the barley cultivar Mans Mink that accumulated three times the normal amount of proline in the soluble fraction of the leaf. Genetic analysis of crosses among the mutants and Golden Promise indicated that salt resistance was controlled by a single semi-dominant gene. Growth of the mutant R5201 was less inhibited than that of Maris Mink by NaCl concentrations of $<100 \text{ mol m}^{-3}$, but at higher concentrations growth was inhibited to the same extent. Mutants BHp10 and Bhp31 were superior to the wheat variety HD1553 and may be of use in breeding for salt tolerance (Kumar and Yadav, 1983).

In *Arabidopsis* mutants that were more resistant to salt than the parent line were identified by Saleki *et al.* (1993). Salt-hypersensitive mutants at the *SOS1*, *SOS2* and *SOS3* loci on chromosome 2 of *Arabidopsis* were defective in the high-affinity K^+ uptake mechanism, and unable to grow at low K^+ concentrations in the medium, especially at low Ca^{2+} levels (Liu and Zhu, 1997a, 1998; Wu *et al.*, 1996; Zhu *et al.*, 1998).

20.3.5.2 Somaclonal Variation

Tissue and cell culture techniques are useful in a variety of areas. They can be used for selection from existing variation, and as a means of generating new variation. Plants with increased resistance to salt can be regenerated from resistant cell lines (Wang and Bao, 1998; Winicov, 1991, 1996). The problems of translating behaviour *in vitro* into whole plant salt resistance have, however, been discussed by Dracup and others (Dracup, 1991; Dracup *et al.*, 1993). Given this analysis, it is not very surprising that selection of salt tolerant lines in tissue culture and followed by the regeneration of whole plants has been less successful than one might expect. Furthermore, cultured cells from tolerant plants may not necessarily be more tolerant than those of glycophytes.

Cell culture also offers the ability to examine altered metabolism (Galiba *et al.*, 1993), effects of compatible solutes (Tal and Katz, 1980), gene expression (Winicov and Shirzadegan, 1997) and cell differentiation (Yen and Yen, 1999) at levels of organization lower than that of the whole plant. Regeneration of whole plants from embryo culture (embryo rescue of wide crosses), transformed cells and pollen grains (to produce haploid and doubled haploid lines) are also important in breeding for salt tolerance. A detailed analysis of these techniques is beyond the scope of this chapter. For detailed reviews of cell and tissue culture and salinity see Gulati and Jaiwal (1997), Hasegawa *et al.* (1994), Tal (1994) and Winicov and Bastola (1997).

20.3.6 HERITABILITY

Having established the existence of useful variation for salt resistance and its components, the next question is how heritable is this variation. Variation that is not inherited in a reasonably predictable way is of little use in a breeding program. This is often addressed using quantitative genetics (Falconer, 1981) to calculate broad- and narrow-sense heritabilities (Table 2) from the performance of parents and progeny of crosses - often complex diallel crosses between several cultivars. These also provide information about additive and dominant inheritance, combining ability of particular lines and environmental effects (Ashraf, 1994). Such studies allow an estimation of the likely benefits of selection.

Although heritability is often quite high (Table 2), exploitation of variation and inheritance in crop breeding requires a long-term breeding program, preferably targeted at specific local conditions. Detailed breeding strategies are beyond the scope of this chapter. For a detailed discussion of breeding techniques see Chaubey and Senadhira (1994).

20.4 A comparative physiologist's perspective

The physiologist's view of plant responses to salinity is dominated by several apparent contradictions. Firstly the majority of halophytic species accumulate relatively high concentrations of Na^+ and Cl^- . Indeed simple calculations show that such accumulation makes a major contribution to essential osmotic adjustment and turgor generation. However many comparative studies on the tolerance of cultivars or closely related species found tolerance, either in terms of vegetative growth or final yield, to be strongly correlated with the ability to **exclude** Cl^- or Na^+ from the shoots (e.g. Rashid *et al.*, 1999; Salam *et al.*, 1999). Secondly, increasing external NaCl salinity causes, in most glycophytes and halophytes, a marked decline in shoot K^+ concentrations. Nevertheless comparative studies, such as those noted above, revealed that tolerance is frequently correlated with the maintenance of a high K^+/Na^+ ratio especially in the younger tissues. Paradoxically the selectivity for K^+ uptake in halophytes is actually enhanced by increasing salinity. The likely cell physiological explanations for such findings are described in Chapter 8.

These contradictions highlight a set of issues in breeding for salt tolerance. It is apparent that even within halophytes or glycophytes, tolerance is not necessarily achieved in identical ways. An obvious example would be the control of leaf salt loads. Salt glands may function in some species, expendable salt bladder cells in others, enhanced succulence in yet others and in some an element of phloem retranslocation of Na^+ or Cl^- . More generally there is a tendency in many species for salt to gradually accumulate in the older leaves, often inducing premature senescence and leaf death, while the meristematic tissues continue to grow relatively normally. Sometimes people speak of "plants being able to grow away from salinity". In the same vein, there is compelling evidence that a number of mechanisms can and do contribute to the

regulation of the comparative transport of K^+ , Na^+ and Cl^- into the roots and of translocation to the individual leaves. It is sufficient to note here that these can include selective uptake and export at the plasma membranes of epidermal cells, regulation of

TABLE 2. Heritability of traits associated with salt resistance.

Species	Character	Heritability		References
		broad sense	narrow sense	
<i>Agrostis stolonifera</i>	tolerance, seedling root growth	0.82-0.94	0.32, 0.28	Ashraf <i>et al.</i> , 1986a,b
<i>Agrostis castellana</i>	seedling root growth		0.26	Ashraf <i>et al.</i> , 1986b
<i>Holcus lanatus</i>	tolerance, seedling root growth	0.82-0.94	0.41, 0.29, 0.19	Ashraf <i>et al.</i> , 1986a,b
<i>Dactylis glomerata</i>	tolerance, seedling root growth	0.82-0.94	0.52, 0.56, 0.32	Ashraf <i>et al.</i> , 1986a,b
<i>Festuca rubra</i>	tolerance, seedling root growth	0.82-0.94	0.29, 0.44	Ashraf <i>et al.</i> , 1986a,b
<i>Lolium perenne</i>	seedling root growth		0.33	Ashraf <i>et al.</i> , 1986b
<i>Puccinellia distans</i>	seedling root growth		0.72	Ashraf <i>et al.</i> , 1986b
<i>Brassica napus</i>	seedling root growth		0.74	Ashraf <i>et al.</i> , 1987
<i>Trifolium alexandrinum</i>	seedling root growth		0.50	Ashraf <i>et al.</i> , 1987
<i>Medicago sativa</i>	seedling root growth		0.52	Ashraf <i>et al.</i> , 1987
<i>Trifolium pratense</i>	seedling root growth		0.98	Ashraf <i>et al.</i> , 1987
<i>Sorghum bicolor</i>	seedling root growth	0.82	0.51	Azhar and McNeilly, 1988
<i>Triticum aestivum</i>	grain weight		0.75, 0.86	Ahsan <i>et al.</i> , 1996
	ion content		0.70 to 0.95	
<i>Hordeum vulgare</i>	germination	0.99	0.75	Mano and Takeda, 1997a,b
diallel crosses	leaf injury index	0.85	0.75	Kebebew and McNeilly, 1996
<i>Pennisetum americanum</i> diallel crosses	tolerance	high		Rao and McNeilly, 1999
<i>Zea mays</i>	seedling tolerance	0.70	0.40	Garcia <i>et al.</i> , 1997
<i>Oryza sativa</i>	shoot Na^+ concentration		0.42, 0.43	
	shoot K^+ concentrations		0.46, 0.52	
	Na^+/K^+ ratio		0.36, 0.40	
<i>Oryza sativa</i> diallel crosses*	Na^+/K^+ ratio	low		Gregorio and Senadhira, 1993
<i>Oryza sativa</i>	tolerance	0.49, 0.83		Jones, 1986; Jones and Stenhouse, 1984
<i>Oryza sativa</i> diallel crosses*	shoot Na^+ accumulation		0.49	Lee <i>et al.</i> , 1996
<i>Oryza sativa</i>	plant height, dry weight, K^+ content, Na^+/K^+ ratio, Na^+ content	0.60-0.81		Won <i>et al.</i> , 1992
<i>Lycopersicon</i>	total fruit weight	0.53		Asins <i>et al.</i> , 1993
	fruit number	0.73		
<i>Lycopersicon</i>	shoot dry weight		0.49	Foolad, 1996

* high g x e effects reported

trans-root flows including the provision of one or more Casparian strips, the specificity of xylem loading, salt extraction from the xylem flow by specialized cells at various cytological levels, as well as ion distribution between cell types in leaves.

Clearly salt tolerance in higher plants is both a product of specific cellular adaptation AND of detailed whole plant physiology AND the integrated function of anatomy, cytology and tissue 'plumbing'. Mechanisms that might be appropriate in one tissue (e.g. Na^+ exclusion in root cortical cells) may not be useful in other tissues (leaves - see the description of the Oertli effect, Chapter 8). Thus control of expression of genes may be as important as the function of the genes themselves.

Broad generalizations have been made seeking to describe specific 'physiotypes' with characteristic suites of adaptive mechanisms (Albert and Kinzel, 1973; Choo and Albert, 1997). Certainly there are valid generalisations about the suites of mechanisms observed in monocotyledonous and dicotyledonous species. Broadly higher Na^+ and Cl^- levels and greater salt-induced succulence are found in dicots, while many Poaceae tend to maintain higher leaf K^+/Na^+ ratios and display limited succulence. This has led to the concept of relatively "salt accumulating" and "salt excluding" species. However this simplification has many drawbacks. In relation to water flow through the transpiration stream, all plants, even those exhibiting limited tolerance, are necessarily salt excluders (otherwise they would soon resemble Lot's wife! Bible: Genesis, Chapter 19, verse 26). Similarly in all species, ions (mainly K^+ , Na^+ , and Cl^-) dominate osmotic adjustment to the external salinity. In this respect a clear distinction can be made with drought, as osmotic adjustment in this latter case may be mainly due to an increased concentration of organic solutes.

This brief precis leads to several important conclusions for any breeding program. Firstly, not only is salinity a complex multi-faceted phenomenon in the field but tolerance is clearly multigenic. Furthermore, selection can be influenced by plant age (Wilson *et al.*, 2000), substrate (Pecetti and Gorham, 1997), nutrition (e.g. silicon supply in hydroponic culture, Yeo *et al.*, 1999), transpiration rate etc. and a wide range of genotype x environment interactions. Secondly the balance of mechanisms involved differs from species to species. Perhaps we should not speak of 'salt tolerance' as if it were a single phenomenon but of 'salt tolerance mechanisms'. Thirdly it is probable that a number of traits needs to be in place consecutively to achieve a major increase in tolerance - this is often referred to as pyramiding (Akbar *et al.*, 1986a; Flowers and Yeo, 1995; Flowers *et al.*, 2000; Gorham and Wyn Jones, 1990; Yeo *et al.*, 1990). Fourthly, simply selecting for given mechanism at the cellular level may well be inadequate.

This rather daunting analysis does, however, have a silver lining. In the last three decades our knowledge of the precise mechanisms relevant to important crop species has grown substantially. Consequently there are real prospects that a co-ordinated effort by breeders, physiologists and molecular biologist to identify important traits, to link them to genetic markers and pyramid them in dedicated breeding programs has a good prospect of success. Success might be more readily achieved if more attention were paid to modeling the integration of the various aspects of vegetative growth,

photosynthesis and water flows and ion accumulation and transport that together control overall salt tolerance.

To cite one example, Garcia *et al.* (1995) developed a multiple rice cross (IR59462) aimed at combining donors for physiological traits (IR4630-22-2-5-1 and IR10167-129-3-4) with the salinity resistance of traditional tall landraces (Nona Bokra and Pokkali) into an agronomically acceptable semidwarf/intermediate plant type. Conventional selection for agronomic characters in early generations selected against low sodium-transporting genotypes. In contrast, mild early selection for low sodium transport enriched the population in potentially salt-resistant genotypes but did not select against semidwarf/intermediate genotypes. It was concluded that selection for agronomic traits should be made after selection for salt resistance and preferably after the population has reached near-homozygosity.

20.5 A molecular biologists perspective

The recent explosive growth of molecular biological studies offers many new avenues for exploring the responses of plants to salinity, and these have been reviewed by Cushman and Bohnert (2000), Flowers *et al.* (1997), Grover *et al.* (1993, 1998, 1999), Jain *et al.* (1997) and Yeo (1998) and in Chapters 17 and 22. This rapid progress, and the continuing development of new molecular techniques, suggests that any review of this area will be outdated soon after, if not before, publication, and the reader should consult the most recent available literature. Some of the main areas are summarized briefly below.

20.5.1 GENES CONCERNED WITH ION TRANSPORT PROCESSES

Physiological studies suggest that control of ion compartmentation and transport at both the cellular and tissue levels are important in salt resistance, but that we know little of the detailed operation, integration and genetics of transport mechanisms. Much of what we do know comes from work on isolated protoplasts and membrane vesicles, and from comparisons with mechanisms in yeast (see Chapters 18, 19 and 21). Table 3 gives some examples of genes that are associated with the membrane transport of Na^+ , K^+ and Ca^{++} .

There is still considerable uncertainty, however, about where and when these genes are expressed, and how their products contribute to net ion movements both at the cellular and whole plant levels. There is considerable scope for comparative studies of the expression and function of similar genes in plants that differ in salt resistance.

20.5.2 GENES INVOLVED IN THE SYNTHESIS OF COMPATIBLE SOLUTES

A range of compatible solutes, *i.e.* solutes which can make a significant osmotic contribution in the cytoplasm without deleterious effects on metabolism, have been described in lower and higher plants. This topic is considered in Chapter 9 and in

several recent reviews (Bartels and Nelson, 1994; Hayashi and Murata, 1998; Huang *et al.*, 2000; McCue and Hanson, 1990; Nuccio *et al.*, 1999; Sakamoto and Murata, 2000; Takabe *et al.*, 1998), and will not be discussed in detail here. Table 4 lists some of the genes involved in the synthesis of these compatible solutes (also, but not very usefully, called osmolytes) and their use in transformation experiments.

In many cases increased concentrations of the compatible solutes have been reported in transformed plants, which are generally more resistant to salinity or other abiotic stress than the untransformed parents.

20.5.3 QTL ANALYSIS AND MARKER-ASSISTED SELECTION (MAS)

Transformation is a very useful tool for molecular geneticists and physiologists, but its long-term contribution to practical crop breeding awaits the outcome of current debates about the safety, release and socio-economic implications of Genetically Modified Organisms (GMOs). Less controversial is the use of molecular markers (RAPDs, RFLPs, AFLPs, microsatellites etc.) to locate (map) genes contributing to quantitative traits, and the use of such information as a selection method (marker-assisted selection, MAS) in conventional plant breeding. Quantitative Trait Loci (QTL) are chromosomal regions where a statistical association (e.g. high LOD score) can be established between molecular markers and quantitative traits such as relative salt tolerance or ion concentrations (Table 5).

TABLE 3. Genes controlling ion transport in relation to salinity

Plant	gene	effect	References
Yeast	HAL1	modulator of cation transport, improves salt tolerance in transgenic cucumber and tomato	Bordas <i>et al.</i> , 1997; Gisbert <i>et al.</i> , 2000; Serrano <i>et al.</i> , 1999a,b; Chapter 21
<i>Arabidopsis</i>	AtKUP1	dual affinity K ⁺ transporter	Fu and Luo, 1998; Kim <i>et al.</i> , 1998
<i>Arabidopsis</i>	CAX1	H ⁺ /Ca ⁺⁺ antiporter	Hirschi <i>et al.</i> , 1996
<i>Arabidopsis</i>	SAL1	regulation of Na ⁺ and Li ⁺ fluxes	Quintero <i>et al.</i> , 1996
<i>Arabidopsis</i>	AtNHX1	vacuolar Na ⁺ /H ⁺ antiport Overexpression confers salt tolerance	Apse <i>et al.</i> , 1999
<i>Hordeum</i>	HAK1	high affinity K ⁺ transporter	Santa-Maria <i>et al.</i> , 1997
<i>Triticum</i> , <i>Hordeum</i> , <i>Oryza</i> , <i>Arabidopsis</i>	HKT1	high affinity Na ⁺ -coupled K ⁺ transporter,	Schachtman <i>et al.</i> , 1997, Schachtman and Liu, 1999, Rubio <i>et al.</i> , 1995; Gassmann <i>et al.</i> , 1996; Wang <i>et al.</i> , 1998; Schachtman and Schroeder, 1994; Golldack <i>et al.</i> , 1997
<i>Triticum</i>	LCT1	low affinity cation transporter	Schachtman <i>et al.</i> , 1997
<i>Triticum</i>	KNA1	enhanced K ⁺ /Na ⁺ discrimination	Dubcovsky <i>et al.</i> , 1996; Gorham <i>et al.</i> , 1997

TABLE 4. Transformation with genes involved in the synthesis of compatible solutes.

Transformed species	gene (enzyme or solute)	source	references
Tobacco	betA (choline dehydrogenase)	<i>Escherichia coli</i>	Holmstrom <i>et al.</i> , 1994, 2000
Tobacco	betB (betaine aldehyde dehydrogenase)		
Tobacco	betA (choline dehydrogenase)	<i>Escherichia coli</i>	Lilius <i>et al.</i> , 1996
Tobacco	ectA, ectB and ectC (ectoine)	<i>Halomonas elongata</i>	Nakayama <i>et al.</i> , 2000
Tobacco	BADH (glycinebetaine)	spinach	Rathinasabapathi <i>et al.</i> , 1994
Tobacco	BADH (glycinebetaine)	barley	Nakamura <i>et al.</i> , 1995, 1997
Tobacco	CMO (choline mono-oxygenase)	spinach	Nuccio <i>et al.</i> , 1998
Tobacco	TPS1 (trehalose)	yeast	Holmstrom <i>et al.</i> , 1996; Romero <i>et al.</i> , 1997; Serrano <i>et al.</i> , 1999b
Tobacco	mtldh (mannitol)	<i>Escherichia coli</i>	Karakas <i>et al.</i> , 1997
Tobacco	ProBosm, proA (proline)	<i>Escherichia coli</i>	Sokhansandzh <i>et al.</i> , 1997
Tobacco	P5CS (proline)	<i>Vigna aconitifolia</i>	Kishor <i>et al.</i> , 1995
Tobacco	mtlD (mannitol)		Tarczynski <i>et al.</i> , 1992, 1993
Tobacco	s6pdh (sorbitol)	apple	Tao <i>et al.</i> , 1995
Tobacco	imtl (D-ononitol)	<i>Mesembryanthemum crystallinum</i>	Vernon <i>et al.</i> , 1993b; Sheveleva <i>et al.</i> , 1997
Rice	P5CS (proline)	<i>Vigna aconitifolia</i>	Zhu <i>et al.</i> , 1998b
Rice	betA (choline dehydrogenase)	<i>Escherichia coli</i>	Takabe <i>et al.</i> , 1998
Rice	codA (choline oxidase)	<i>Arthrobacter globiformis</i>	Sakamoto <i>et al.</i> , 1998
Rice	BADH (glycinebetaine)	barley	Kishitani <i>et al.</i> , 2000
<i>Synechococcus</i>	betA, betB	<i>Escherichia coli</i>	Nomura <i>et al.</i> , 1998; Takabe <i>et al.</i> , 1998
<i>Arabidopsis</i>	codA (choline oxidase)	<i>Arthrobacter globiformis</i>	Hayashi and Murata, 1998; Hayashi <i>et al.</i> , 1997, 1998; Sakamoto <i>et al.</i> , 2000
<i>Arabidopsis</i>	mtldh (mannitol)	<i>Escherichia coli</i>	Thomas <i>et al.</i> , 1995
<i>Arabidopsis</i> , <i>Brassica napus</i> , Tobacco	COX (choline oxidase)	<i>Arthrobacter pascens</i>	Huang <i>et al.</i> , 2000

For example, in tetraploid wheat, QTL analysis of K^+/Na^+ ratios in young leaves of greenhouse (hydroponic) and saline-field-grown plants of chromosome 4B/4D recombinant lines (Dvorak *et al.*, 1994; Dubcovsky *et al.*, 1996) showed very high LOD scores in the *Kna1* region for wheat plants grown in hydroponics, but lower values for plants grown in the field. Subsequent RFLP analysis of 129 families (Dubcovsky *et al.*, 1996) confirmed that the trait was controlled by a single gene (*Kna1*) which was completely linked to 5 markers (*Xwg199*, *Xabc305*, *Xbcd402*, *Xpsr567* & *Xpsr375*) on the distal third of the long arm of chromosome 4D. To remove the distal 4D genetic material from the *Kna1* recombined chromosomes, and to further

TABLE 5. Identified molecular markers and QTL for traits related to salt resistance.

Species	Population	Traits	References
Maize	inbred lines	growth and yield QTL	Abdel <i>et al.</i> , 1997
Tomato	<i>L. pimpinellifolium</i> interspecific hybrid	fruit weight and number QTL	Breto <i>et al.</i> ,
Tomato	<i>L. pimpinellifolium</i> interspecific hybrid, BC1	map construction	Chen and Foolad, 1999
Tomato	<i>L. pennellii</i> interspecific hybrid, BC1F6	mapping and introgression	Eshed <i>et al.</i> , 1992
Tomato	<i>L. pennellii</i> interspecific hybrid, F2	tolerance QTL	Foolad and Jones, 1993; Foolad <i>et al.</i> , 1997
Tomato	<i>L. pimpinellifolium</i> interspecific hybrid, BC1F1	germination, growth QTL	Foolad, 1999; Foolad <i>et al.</i> , 1998, 1999a,b; Foolad and Chen, 1999
Tomato	<i>L. pimpinellifolium</i> and <i>L. cheesmannii</i> interspecific hybrids	salt tolerance and fruit number QTL	Monforte <i>et al.</i> , 1997,1999
Rice	wild and cultivated species	RAPD markers linked to tolerance	Farooq <i>et al.</i> , 1995
Rice	F7 RILs*	ion accumulation QTL	Flowers <i>et al.</i> , 2000
Rice	F8 RILs	ion accumulation, resistance scores, water content QTL	Aktar and Gorham, unpublished
Rice	F7 RILs	salt tolerance QTL, chromosome 5	Lin <i>et al.</i> , 1997, 1998
Rice		salt tolerance QTL	Mackill <i>et al.</i> , 1999
Rice	doubled haploids	germination, seedling root growth, seedling root length and vigour QTL	Prasad <i>et al.</i> , 2000
Rice	parents and mutants; F2	RFLP markers for salt tolerance	Zhang <i>et al.</i> , 1994,1995a
Rice		H ⁺ -ATPase, chromosome 12	Zhang <i>et al.</i> , 1999
Barley	<i>H. spontaneum</i> accessions	salt tolerance markers	Forster <i>et al.</i> , 1997, 2000
Barley	doubled haploids	germination and seedling tolerance QTL	Mano and Takeda, 1997a,b
Wheat	homoeologous recombinant lines	mapping of enhanced K ⁺ /Na ⁺ discrimination (KNA1) on chromosome 4	Luo <i>et al.</i> , 1996a,b; Dubcovsky <i>et al.</i> , 1996
citrus	BC1	Na ⁺ and Cl ⁻ accumulation, salt tolerance and morphology QTL	Tozlu <i>et al.</i> , 1999a,b
soybean	tolerant and sensitive cultivars	markers for salt tolerance	Zhong <i>et al.</i> , 1997

* RILs - recombinant inbred lines

map the *Kna1* locus, two lines were subjected to another round of homoeologous recombination in the absence of the *PH1* locus (Luo *et al.*, 1996b). By this procedure, *Kna1* was mapped in a 1.1 cM region on the 4B/4D map spanning markers *Xmwig2112* and *Xpsr375*. This region was also mapped in *T. monococcum*.

QTL analysis is usually performed on a population (usually > 100) of hybrid lines (F₂, recombinant inbred lines from about F₆, backcross lines or doubled haploid lines) in which a large number of molecular markers are mapped. Development of suitable hybrid lines requires parents that differ substantially in the quantitative trait under consideration, and the whole process is time-consuming and expensive. There are also serious problems with genotype x environment interactions. Choosing an appropriate trait is an important step in QTL analysis, and in the case of salt resistance requires

some knowledge of the basic physiology of the species. On the other hand, MAS is very useful in pyramiding several different traits or trait loci.

20.5.4 FUNCTIONAL GENOMICS

There are two complementary approaches to understanding the responses of plants to salinity. These may be summarized as 1) Identify a physiological, morphological or developmental trait and then find the gene(s), or 2) Find a gene and then work out what it does in the whole plant. Cushman and Bohnert (2000) have predicted that progress will be made in comparative genomic studies of a diverse set of model organisms, and through the use of a variety of molecular techniques. Specifically, 'The discovery of novel genes, determination of their expression patterns in response to abiotic stress, and an improved understanding of their roles in stress adaptation (obtained by the use of functional genomics) will provide the basis of effective engineering strategies leading to greater stress tolerance'. Functional genomics, in the sense of determining gene expression (as mRNA or proteins) can, however, only identify genes whose expression is altered by stress treatments. It does not usually provide information about constitutive differences that lead to differences in salt tolerance. Nevertheless the contributions of functional genomics (including differential display, e.g. Tsukatani *et al.*, 1999) and experiments involving complementation of yeast mutants (e.g. Matsumoto *et al.*, 1997; Peng *et al.*, 1995), have been impressive over the last decade (Table 6). Earlier work, including altered protein synthesis, was reviewed by Hurkman (1992).

20.6 Where now?

This brief account of the genetics of the responses of plants to salinity has, we hope, brought together different views of the problems, their complexity, and possible routes to crops with increased resistance to salinity. It seems clear that no one discipline has all the answers, but that each can make a contribution either to understanding the mechanisms of resistance, and/or to practical breeding strategies.

At one end of the scale greater consideration needs to be given to the requirements of users and growers in target environments. The need for involvement of the farmer and consumer in selection of salt-resistant varieties is important because an acceptable new variety needs to be as good as or better than currently grown varieties in terms of pest and disease resistance, consumer acceptability and marketing, processing quality *etc.* Since these requirements are geographically specific, no one variety will satisfy all needs.

At the other end of the scale, molecular biology offers exciting new possibilities (Winicov, 1998; Zhu *et al.*, 1997) and techniques (Pardo *et al.*, 1998; Pardo *et al.*, 1996). In particular, understanding of stress signaling and regulation of gene

TABLE 6. Genes identified using functional genomics and related techniques.

Species	gene	function, product or notes	references
alfalfa	Alfin1	regulates the inducible gene MsPRP2	Bastola <i>et al.</i> , 1998a,b; Winicov, 2000
alfalfa	Alfin1, MsPRP2, pA18	proline-rich cell wall protein	Bastola <i>et al.</i> , 1998a,b; Deutsch and Winicov, 1995; Winicov and Bastola, 1999; Winicov and Shirzadegan, 1997
alfalfa	MsP5CS-1	Delta(1)-pyrroline-5-carboxylate synthetase (P5CS)	Ginzberg <i>et al.</i> , 1998
alfalfa	MsP5CS-2	Increased transcription in salt-tolerant cell lines	Winicov and Krishnan, 1996
tomato	rbcS, cab1, cab4, alfin1, pA18, H3cl, H3cll	peroxidase	Botella <i>et al.</i> , 1994
tomato	TPX1	S-adenosyl-L-methionine synthetase	Espartero <i>et al.</i> , 1994
tomato	SAM1, SAM2, SAM3	Delta(1)-pyrroline-5-carboxylate synthetase (P5CS)	Fujita <i>et al.</i> , 1998
tomato	tomPRO2	lipid transfer protein?	Torres-Schumann <i>et al.</i> , 1992
rice	HKT	high-affinity K ⁺ transporter, or Na ⁺ transporter	Golldack <i>et al.</i> , 1997
rice	SalT	affects Na ⁺ accumulation?	Claes <i>et al.</i> , 1990; Roy <i>et al.</i> , 1993
rice	SalT, dhm4	inducible by proline metabolites	Iyer and Caplan, 1998
rice	sodA1, sodCc2, sodCp	Mn-superoxide dismutase	Kaminaka <i>et al.</i> , 1999
rice	SAMDC1, REF1A	cytosolic Cu/Zn-superoxide dismutase	Li <i>et al.</i> , 1999
rice	transcripts	plastidic Cu/Zn-superoxide dismutase	Minhas and Grover, 1999
rice	osr40g2, osr40g3, oslea3	S-adenosylmethionine decarboxylase, translation elongation factor Iri	
rice	OsBZ8	triose phosphate isomerase, aldolase, glyceraldehyde phosphate dehydrogenase, pyruvate kinase	
rice	OsCDPK7	35 salt-induced polypeptides and 17 salt-repressed polypeptides	Ramani and Apte, 1997
rice	SIGR1 to 13	unknown	Moons <i>et al.</i> , 1995, 1997a,b
rice	rbcL	group 3 LEA protein	
rice		DNA-binding, bZIP protein	Nakagawa <i>et al.</i> , 1996
rice		Ca ⁺⁺ -dependent protein kinase	Saijo <i>et al.</i> , 2000
rice		Salt-Induced Gene in Rice, some of which are homologous to Rab16	Zhang and Chen, 1996
rice		truncated large subunit of Rubisco	Zhang <i>et al.</i> , 1995

Species	gene	function, product or notes	references
rice	rbcl, Salt, rab16	truncated large subunit of Rubisco, Na ⁺ accumulation, and ABA-responsive genes	Zhang <i>et al.</i> , 1996
rice	OSA3	plasma membrane H ⁺ -ATPase	Zhang <i>et al.</i> , 1999
rice and finger millet	rab16A, M3	LEA proteins	Jayaprakash <i>et al.</i> , 1998
barley, transgenic rice	HVA1	barley LEA gene HVA1 increases salt tolerance in transgenic rice	Xu <i>et al.</i> , 1996
<i>Arabidopsis</i>	AtPLC1	phosphatidylinositol-specific phospholipase C	Hirayama <i>et al.</i> , 1995
<i>Arabidopsis</i>	ARSK1	root-specific protein kinase	Hwang and Goodman, 1995
<i>Arabidopsis</i>	ATCP1	cytosolic root and flower Ca ⁺⁺ -binding protein	Jang <i>et al.</i> , 1998
<i>Arabidopsis</i>	p5cs, rab18, ltu78	p5cs and unknown proteins	Knight <i>et al.</i> , 1997
<i>Arabidopsis</i>	rd29A, DREB1A, DREB2A	LEA-like hydrophilic protein	Liu <i>et al.</i> , 2000
<i>Arabidopsis</i>	AHAL3	DREB transcription factors	Espinosa Ruiz <i>et al.</i> , 1999
<i>Arabidopsis</i>	DREB2A, DREB2B	flavoprotein homologue of HAL3 of yeast	Nakashima <i>et al.</i> , 2000
<i>Arabidopsis</i>	APDH	DRE/CRT-binding proteins	Peng <i>et al.</i> , 1996
<i>Arabidopsis</i>	AGSK1	proline dehydrogenase induction by proline inhibited by salinity	Piao <i>et al.</i> , 1999
<i>Arabidopsis</i>	ESTs	GSK3/shaggy-like protein kinase	Plh <i>et al.</i> , 1997
<i>Arabidopsis</i>	SAL1	15 salt-inducible ESTs, 5 identical to previously identified genes	Quintero <i>et al.</i> , 1996
<i>Arabidopsis</i>	At-PSS, At-P5S	3'(2')-5'-biphosphate nucleotidase and inositol polyphosphate 1-phosphatase	Savoure <i>et al.</i> , 1997
<i>Arabidopsis</i>	AtP5CS1, AtP5CS2	proline synthesis	Strizhov <i>et al.</i> , 1997; Szabados <i>et al.</i> , 1998
<i>Arabidopsis</i>	Atmyb2	DNA-binding	Urao <i>et al.</i> , 1993
<i>Arabidopsis</i>	ATCDPK1, ATCDPK2	Ca ⁺⁺ -dependent, calmodulin-independent protein kinases	Urao <i>et al.</i> , 1994
<i>Arabidopsis</i>	rd29A, rd29B, rd22	cis-acting elements	Iwasaki <i>et al.</i> , 1995; Yamaguchi-Shinozaki and Shinozaki, 1994
<i>Arabidopsis</i>	cor6.6, kin1	transcriptional regulation, induced by cold and osmotic stresses	Wang <i>et al.</i> , 1995
<i>Arabidopsis</i> sos1 mutant	P5CS, AtMYB	P5CS, transcriptional factor	Liu and Zhu, 1997
wheat	WESR1, WESR2, WESR3, WESR4, WESR5	Wheat Early Salt-stress Responding genes. WESR5 homologous with glucose-6-phosphate dehydrogenase	Nemoto <i>et al.</i> , 1999
wheat	TaSAMDC	S-adenosylmethionine decarboxylase	Li and Chen, 2000
wheat	PKABAI	ABA-responsive protein kinase	Holappa and Walker-Simons, 1995
wheat	EmBP-1 homologue	ABA-responsive element transcription factor	Gupta <i>et al.</i> , 1998
<i>Lophopyrum</i>	dhn1, HmGlt4, HmAPt2.2,	dehydrin, lipid transfer proteins, translation elongation factor	Tabaei-Aghdaei <i>et al.</i> , 2000;

Species	gene	function, product or notes	references
<i>elongatum, Agropyron desertorum</i>	blt63, unidentified genes		Gulick and Dvorak, 1992
mustard	msc1, msc2	unknown	Gurjar and Roy, 1994
transformed plants	DREB1A	transcription factor that interacts with the dehydration responsive element (DRE) to regulate gene expression in response to stress	Kasuga <i>et al.</i> , 1999
sugar beet	partial genomic clones	Beta V-ATPase 70 kDa subunit and	Lehr <i>et al.</i> , 1999
sugar beet and <i>Amaranthus caudatus</i>	BVA/70 and BVA/16-1	Beta V-ATPase 16 kDa subunit, including promoter regions	Russell, <i>et al.</i> , 1998
<i>Mesembryanthemum crystallinum</i>	CMO	choline monooxygenase (glycinebetaine synthesis)	Forsthoefel <i>et al.</i> , 1995
<i>Mesembryanthemum crystallinum</i>		cofactor-independent phosphoglyceromutase	Low <i>et al.</i> , 1996; Tsiantis <i>et al.</i> , 1996
<i>Mesembryanthemum crystallinum</i>	Ppc1, Imt1, B5, Gpd1	vacuolar H ⁺ -ATPase subunits	Vernon <i>et al.</i> , 1993
<i>Mesembryanthemum crystallinum</i>		salt-induced biochemical pathways	Meyer <i>et al.</i> , 1990
<i>Mesembryanthemum crystallinum</i>		The authors estimated that > 100 genes are differentially regulated by salt stress	Schaeffer <i>et al.</i> , 1995
<i>Mesembryanthemum crystallinum</i>	Ppc1, Gap1	CAM-specific isozyme of PEP carboxylase, NAD-dependent	Mizrahi <i>et al.</i> , 1997
jojoba	cDNA clones	glyceraldehyde-3-phosphate dehydrogenase	Perez-Prat <i>et al.</i> , 1994
tobacco		12 clones down-regulated and 19 clones up-regulated by salt treatment	Yamada <i>et al.</i> , 1997
<i>Nicotiana excelsior</i>	NeMip1, NeMip2, NeMip3	H ⁺ -ATPase mRNA	Yamada <i>et al.</i> , 2000
<i>Nicotiana species</i>	NpAldP2, NaAldP2, NeAldP2	water channel proteins	Redkar <i>et al.</i> , 1996
<i>Aspergillus</i>		plastidic fructose-1,6-bisphosphate aldolase	Holland <i>et al.</i> , 1993
citrus		8 genes induced by adaptation to salt, including mitochondrial ATPase subunits, transport protein and a ubiquitin-extension protein	Fisher <i>et al.</i> , 1994
<i>Dunaliella</i>		homologue of mammalian glutathione peroxidases	Uno <i>et al.</i> , 1998
<i>Aster tripolium</i>	Samip A, Samip B, Samip C	60 kd plasma membrane protein	
		aquaporin homologues	

expression should help to integrate apparently unrelated physiological and biochemical responses to salinity and other abiotic stresses. On the other hand, the limitations of molecular techniques should be recognized. Many of the studies currently operate at a naïve level in relation to whole plant phenology and physiology. QTL analysis in relation to salinity is still at an early stage in terms of mapping resolution and gene identification, and the problem of genotype x environment interactions needs to be resolved before marker-assisted selection can be considered as a practical tool for plant breeders. Finally, the fate of transgenics (GMOs) in agriculture may well be decided on the basis of consumer acceptability.

20.7 References

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CHAPTER 21

HALOTOLERANCE GENES IN YEAST

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Abstract

Yeast molecular genetics has unraveled some novel aspects which may be relevant to plant tolerance to salt stress. The plasma membrane electrical potential drives the uptake of toxic cations such as sodium. This biophysical parameter is modulated by several protein kinases acting on the proton pumping ATPase and on the potassium transporter. Salt stress signaling occurs by two mechanisms involving turgor loss (Hog1 pathway) and calcium (calcineurin pathway). Hog1 phosphorylates and inactivates the Sko1 transcriptional repressor. Calcineurin dephosphorylates and activates the Crz1/Hal8 transcriptional activator. Sodium inhibits a 3',5'-bisphosphate nucleotidase (Hal2) involved in sulfate metabolism and RNA processing

21.1 Introduction

Halotolerance genes are those encoding important determinants of salt tolerance in a particular organism. In the present chapter I will describe these genes in the model organism *Saccharomyces cerevisiae* (baker's yeast) with the perspective that the strategies utilized for their identification and their physiological roles could be partially extrapolated to higher plants.

Halotolerance genes may encode proteins involved in five different physiological activities (Figure 1). Salt stress consists of both osmotic stress (due to the increased concentration of extracellular solutes and concomitant turgor loss) and intracellular ion toxicity. Therefore, two primordial types of halotolerance proteins correspond to targets of turgor loss and ion toxicity. Cell growth depends on turgor-driven stretching of the cell wall and the protein machinery sensing this physical force could be a target of salt stress. Ion toxicity targets could correspond to essential intracellular enzymes directly inhibited by salt. As indicated in the figure, salt directly impinges on turgor targets while it needs to be transported into the cell to reach ion toxicity targets.

Osmotic stress is counteracted by osmolyte synthesis while ion toxicity is relieved by transport systems at either the plasma or vacuolar membranes. Osmolytes or compatible solutes are small organic molecules synthesized by cells to counteract the osmotic pressure of the external medium. Molecules such as glycerol, trehalose, betaine or proline are compatible with cellular systems and enzymes involved in their synthesis are important halotolerance determinants.

The response of cells to salt stress includes many regulatory proteins, which participate in several signal transduction pathways. The capability for osmotic adjustment (by osmolyte synthesis) and for ion extrusion from the cytosol may be modulated at the gene and protein levels and the regulatory components of these responses would be important determinants of halotolerance.

The relative importance of osmotic stress and ion toxicities during salt stress depends on the salt concentration and on the relative capabilities of each organism for osmotic adjustment and ion transport, which may be influenced by growth conditions. For example, in the yeast *Saccharomyces cerevisiae* growing on glucose media sodium toxicity is the limiting factor for salt tolerance while in the same organism growing on galactose, a poorer carbon source, osmolyte synthesis is limiting (Garcia *et al.*, 1997).

There are two different approaches to isolate halotolerance genes. The physiological identification of the different types of halotolerance determinants described above may lead to a biochemical approach to isolate the proteins involved in turgor sensing, ion toxicity, osmolyte synthesis, ion transport and in regulation of salt responses. This "phenomenological" approach may be successful in the case of abundant proteins of well-defined function, such as enzymes involved in osmolyte biosynthesis. It has also been instrumental for the isolation of proteins and cDNAs of unknown function induced during salt stress and which could correspond to putative defenses. However, this approach may experience enormous difficulties in the isolation of low-abundance proteins involved in ion transport and in the identification of targets and regulatory components of salt stress. In addition, the physiological relevance of the isolated components cannot be assessed. For example, an isolated enzyme which catalyzes osmolyte biosynthesis "in vitro" may not be relevant under "in vivo" conditions because of metabolic constraints in the supply of substrates. Also, a protein induced by salt stress may not be important for defense against this particular stress because there is a considerable lack of specificity in many stress responses (Serrano, 1996).

A mutational analysis consisting on both "gain of function" and "loss of function" approaches may identify important determinants of salt tolerance independently of their abundance in the cell and of their previous physiological characterization (Figure 2). This strategy involves the generation of a collection of mutants on all possible genes and the selection of those exhibiting altered salt tolerance. This "functional" approach assures the physiological relevance of the identified component but it must find the way from the mutant to the underlying gene. This is only straightforward in some model organisms such as *Saccharomyces cerevisiae*. In this yeast it is possible to clone genes by complementation of mutants with a plasmid library. In addition, it is feasible to overexpress genes at random with multicopy plasmid libraries. Once selected, the recovery of plasmids from halotolerance clones

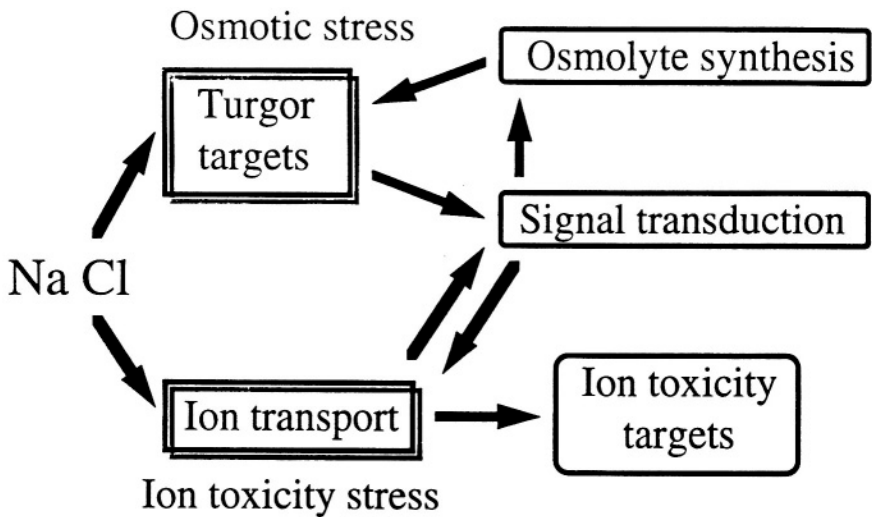


Figure 1. The five kinds of halotolerance determinants: turgor targets, ion transporters, signal transducers, enzymes for osmolyte synthesis and ion toxicity targets. Arrows indicate connections between the different components.

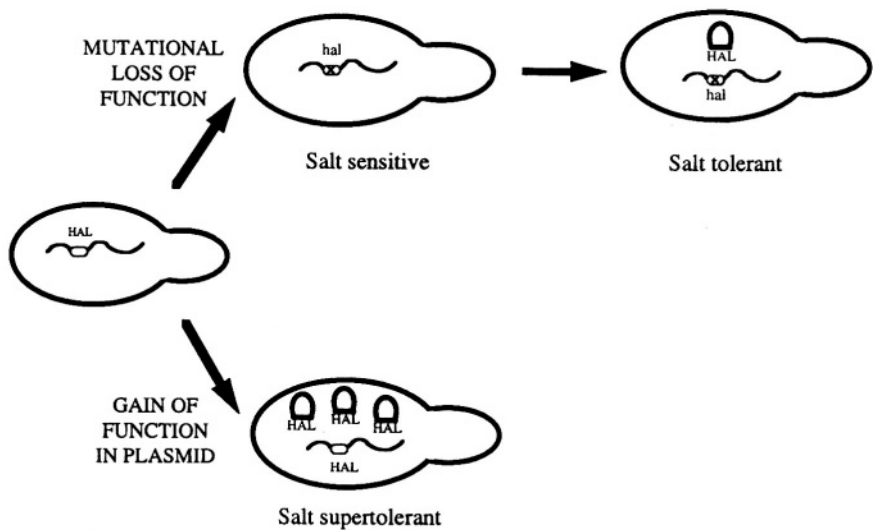


Figure 2. Strategies for identifying halotolerance genes in yeast.

allows the identification of the relevant genes (Guthrie and Fink, 1991). Although similar approaches are becoming available for the model plant *Arabidopsis thaliana*, the most detailed mutational analysis of salt tolerance up to now has been performed in yeast and the resulting picture is described below. We will end with a discussion about the extrapolation of these yeast halotolerance mechanisms to higher plants.

21.2 Ion transporters

The major ion transporters identified in the plasmatic and vacuolar membranes of yeast cells are shown in Figure 3. Pma1 corresponds to the primary H^+ -pumping ATPase, an enzyme essential for yeast growth (Serrano, 1991). On the other hand, the activity of the high affinity K^+ uptake system (encoded by the *TRK1* and *TRK2* redundant genes) is essential at low K^+ concentrations but dispensable in media with high K^+ concentrations (10^{-1} M). In the latter conditions, a non-specific cation leak mediated by several membrane transporters provides the K^+ uptake required for growth (Gaber, 1992; Serrano *et al.*, 1999). The Tok1 outward K^+ channel does not affect salt tolerance (Rios *et al.*, 1997), but all the other plasma membrane systems of Figure 3 function as halotolerance determinants. The major cation extrusion system of *S. cerevisiae* corresponds to the Ena1 ATPase, a non-specific cation-extrusion pump (Haro *et al.*, 1991; Benito *et al.*, 1997). Wild type yeast cells tolerate Na^+ and Li^+ concentrations of the order of 1 and 0.1 M, respectively but mutants devoid of the Ena1 pump are sensitive to concentrations one order of magnitude lower. It must be indicated that Li^+ usually shares transport systems and toxicity targets with Na^+ but, as it is active at lower concentrations, it is useful to differentiate osmotic and ion effects. In the absence of Ena1 other cation extrusion systems become important for salt tolerance such as the Nha1 antiporter (Prior *et al.*, 1996). Another important system is the Snq2 ATPase (Miyahara *et al.*, 1996). There are three types of ion-pumping ATPases: Pma1 and Ena1 belong to the P-type family of enzymes, characterized by a phosphorylated intermediate, while Snq2 belongs to the multidrug resistance or ABC-type (Paulsen *et al.*, 1998). The third type is exemplified by the vacuolar ATPase described below.

Two important transport pathways not yet identified at the molecular level are the non-specific "cation leak" of the yeast plasma membrane and its mechanosensitive ion channel. The "cation leak" participates in the low-affinity uptake of K^+ and toxic cations such as Li^+ , Na^+ , Ca^{2+} , H^+ , hygromycin B, tetramethylammonium etc. and it may represent the additive contribution of many yeast permeases for sugars, amino acids, ammonium and divalent cations which have small, non-specific cation transport activity (Ko *et al.*, 1993; Wright *et al.*, 1997; Liang *et al.*, 1998; Madrid *et al.*, 1998). This leak has been described at the electrophysiological level as a non-selective cation channel partially blocked by divalent cations (Bihler *et al.*, 1998; Roberts *et al.*, 1999). The stretch-activated channel identified at the electrophysiological level (Gustin *et al.*, 1988) may correspond to small proteolipids as in the case of the *Escherichia coli* MscL channel (Sukharev *et al.*, 1994). One important mechanism of salt tolerance recently identified in *S. cerevisiae* involves the modulation of electrical potential by the combined activities of Pma1 (the generator of voltage) and Trk1,2 (the major consumer). Decreased activity of Pma1 or increased activity of Trk1,2 results in

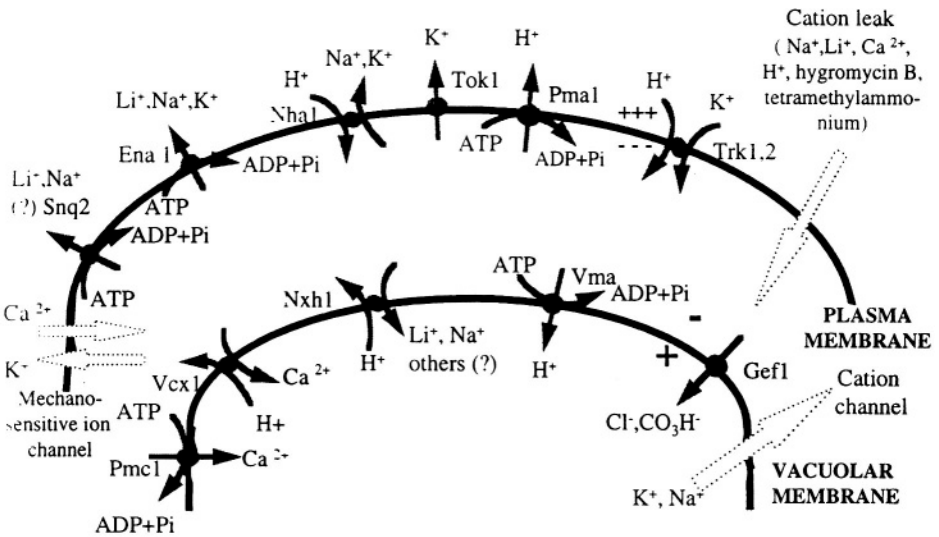


Figure 3. The major ion transporters identified at yeast plasmatic and vacuolar membranes. Those indicated by discontinuous lines have not been identified at the molecular level.

lowering of membrane potential and reduced uptake of toxic cations by the leak pathway (Perlin *et al.*, 1988; Vallejo and Serrano, 1989; Withee *et al.*, 1998; Madrid *et al.*, 1998; Mulet *et al.*, 1999; Goossens *et al.*, 2000). On the other hand, increased activity of Pma1 or decreased activity of Trk1,2 results in hyperpolarization and increased uptake and sensitivity to toxic cations. Interestingly, a conserved plasma membrane proteolipid (Pmp3) has been found to modulate the yeast membrane potential (Navarre and Goffeau, 2000).

The role of vacuolar compartmentation in salt tolerance has been demonstrated by the sensitivity to toxic cations, including Na⁺ and Li⁺, of yeast mutants deficient in the vacuolar H⁺-ATPase (Jones *et al.*, 1997). This complex enzyme belongs to the family of V- and F-type ATPases (Paulsen *et al.*, 1998), distinct from the P- and ABC-type mentioned above. Calcium accumulation into the vacuole is mediated by both the Pmc1 P-type ATPase (Cunningham and Fink, 1994) and by the Vcx1 antiporter (Cunningham and Fink, 1996), while Na⁺ accumulation seems mediated by the Nhx1 antiporter (Nass *et al.*, 1997; Nass and Rao, 1998). The latter is an important determinant of salt tolerance as demonstrated by the salt-sensitive phenotype of *nhx1* mutants. Another important halotolerance gene is *GEF1*, encoding a vacuolar anion channel. Chloride (or bicarbonate) transport is required for vacuolar acidification to

achieve electrical balance during H^+ pumping by the vacuolar ATPase. Accordingly, a defect in this gene leads to sensitivity to toxic cations including Na^+ (Gaxiola *et al.*, 1998). Acidification of the vacuolar compartment seems required for operation of a H^+ -cation antiporter, probably the product of the *NHX1* gene. The mechanism of cation release from the vacuole has not been identified but it may correspond to a channel identified at the electrophysiological level (Bertl and Slayman, 1990).

21.3 Signal transduction

The activities of many yeast transporters described above is regulated at the protein and gene levels by several signal transduction pathways. Components of these pathways have been identified in the search for halotolerance determinants because by modulating ion transporters they dramatically influence the salt tolerance of yeast cells. As expected, a common mechanism of action of many halotolerance proteins is to modulate the major cation extrusion pump of yeast cells encoded by the *ENA1* gene (Figure 4). The promoter of this gene is regulated by a plethora of signal transduction pathways and the details are only known for some of them. High Na^+ concentrations activate the calcineurin pathway, probably by raising intracellular Ca^{2+} levels. Calcineurin is a Ca^{2+} -activated protein phosphatase which induces several yeast genes, including *ENA1* (Nakamura *et al.*, 1993; Mendoza *et al.*, 1994; Marquez and Serrano, 1996), by regulating the nuclear localization of the zinc finger transcription factor Crz1/Tcn1/Hal8 (Stathopoulos-Gerontides *et al.*, 1999). Crz1/Hal8 is a positive regulator of *ENA1* expression and its promoter element (UAS_{CN} in Figure 4) has recently been identified (Mendizabal *et al.*, 2001).

The best-characterized pathways which modulate *ENA1* expression are the glucose catabolic repression pathway and the Hog1 MAP kinase pathway. Glucose starvation in *S. cerevisiae* activates the Snf1 protein kinase which in turns inactivates the Mig1,2 repressor, which interacts with a characteristic negative promoter element (URS_{MIG}) in several genes including *ENA1* (Proft and Serrano, 1999). Osmotic stress, on the other hand, operates via the Sho1 and Sln1 membrane proteins. This pathway involves Hog1 as MAP kinase, Pbs2 as MAP kinase kinase and two different MAP kinase kinases, Ste11 and Ssk2,22 regulated by Sho1 and Sln1, respectively (Wurgler-Murphy and Saito, 1997). When this cascade is activated by osmotic stress, Hog1 inactivates the Sko1 repressor, a leucine zipper transcription factor which blocks *ENA1* expression under normal conditions by binding to a characteristic negative promoter element (Proft and Serrano, 1999), identical to animal cAMP response elements (URS_{CRE}). Therefore *ENA1* expression is controlled by two repressors, Mig1,2 and Sko1, and one activator, Hal8, modulated by such different signals as high Na^+ , glucose starvation and osmotic stress. Although the physiological roles of the high Na^+ and osmotic stress pathways are obvious, it is not clear why the capability for Na^+ extrusion is enhanced upon glucose starvation. Other less well characterized pathways which modulate *ENA1* expression are the protein kinase A pathway activated by nutrients, the Hal3-Ppz1 pathway responding to unknown signals (Nadal *et al.*, 1998), the Ure2-Gln3 pathway activated by ammonium and glutamine starvation (Withee *et al.*, 1998) and an unknown pathway triggered by high pH (Garcia-deblas *et al.*, 1993). Again, the physiological roles of these regulations are not obvious.

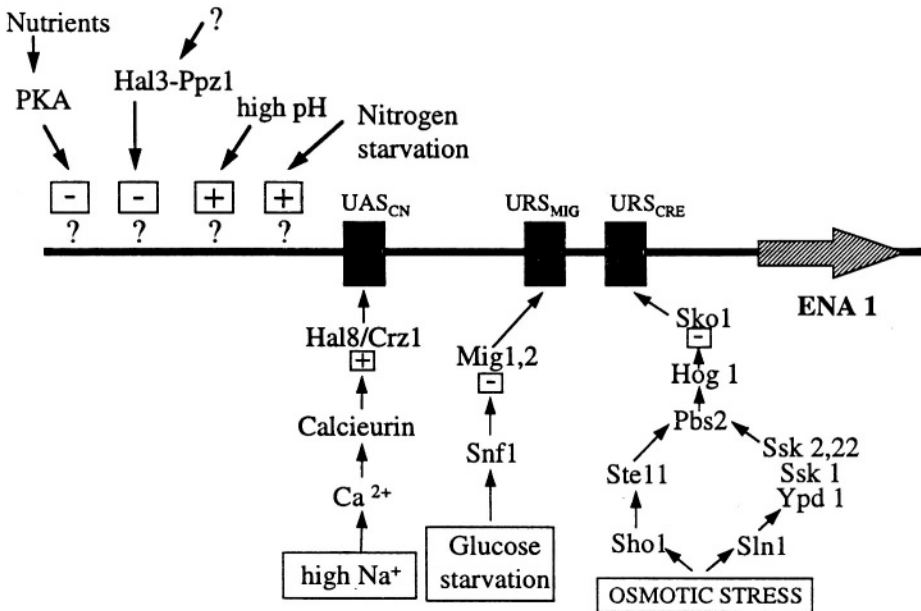


Figure 4. Regulation of the *ENA1* gene encoding the major yeast sodium pump. UAS: positive promoter element (Upstream Activating Sequence); URS: negative promoting element (Upstream Repressing Sequence). The subscripts CN, MIG and CRE indicate that these promoter element are operated by either calcineurin (CN), the Mig1,2 repressor (MIG) or resemble animal cAMP response elements (CRE).

Another important transporter regulated by halotolerance proteins is the Trk1,2 K^+ uptake system (Figure 5). Although the mechanisms of regulation (transcriptional or at the protein level) are unknown, the high Na^+ -calcineurin pathway activates Trk1,2 (Mendoza *et al.*, 1994) and the halotolerance protein Hal1 and the protein kinases Hal4,5 may mediate the activation of Trk1,2 in response to K^+ starvation (Mulet *et al.*, 1999; unpublished observations of J.M. Mulet). As discussed above, activation of Trk1,2 results in decreased membrane potential and reduced uptake of toxic cations including Na^+ . The protein kinase Ptk2, highly homologous to Hal4 and Hal5, has recently been described as a regulator of Pma1 (Goossens *et al.*, 2000).

Many other signal transducing components, including protein kinases and transcription factors, have been described to influence salt tolerance in *S. cerevisiae* (Serrano, 1996) but as their mechanisms have not been elucidated we will not discuss them here.

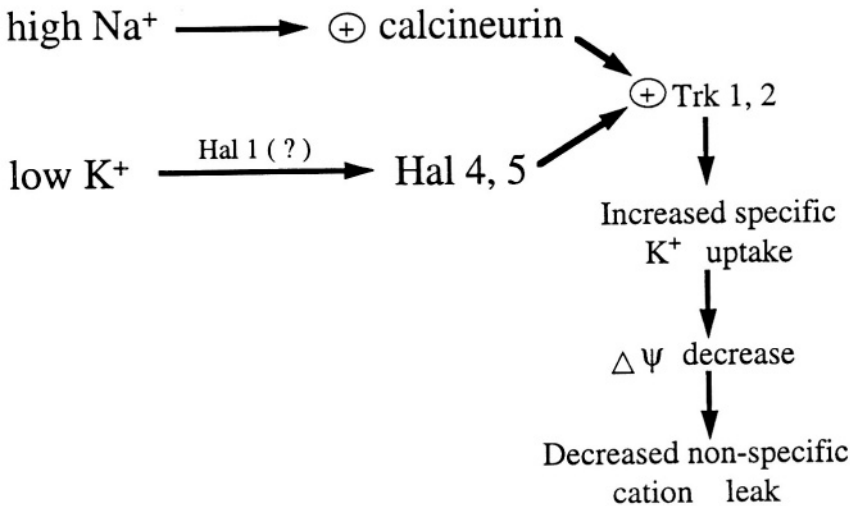


Figure 5. Regulation of potassium transport, electrical membrane potential and cation leak in yeast.

21.4 Sodium toxicity targets

We know little about "in vivo" targets for chloride toxicity but yeast molecular genetics has provided evidence for a physiological target of Na^+ (and Li^+) toxicity: the Hal2 nucleotidase (Glaser *et al.*, 1993; Murguia *et al.*, 1995, 1996; Dichtl *et al.*, 1997). Li^+ and Na^+ are powerful inhibitors of the 3',5'-bisphosphate nucleotidase encoded by the *HAL2* gene, with half-maximal inhibition at 0.1 mM and 20 mM, respectively (Murguia *et al.*, 1995). This phosphatase is required to convert into adenosine-5'-phosphate (AMP) the 3'-phosphoadenosine-5'-phosphate (PAP) generated during the utilization of the activated sulfate of 3'-phosphoadenosine-5'-phosphosulfate (PAPS) (Figure 6). Yeast cells treated with toxic concentrations of Li^+ or Na^+ accumulate PAP because of inhibition of Hal2 nucleotidase (Murguia *et al.*, 1996). This in turn inhibits PAPS utilizing enzymes such as PAPS reductases and sulfotransferases (Murguia *et al.*, 1996) and the rRNA-processing exoribonucleases Rat1 and Xrn1 (Dichtl *et al.*, 1997). In addition, Li^+ (and probably Na^+) directly inhibits the RNA processing ribonuclease MRP (a ribozyme), which also contributes to salt toxicity (Dichtl *et al.*, 1997). More recently, the yeast inositol monophosphatase has been shown to be sensitive to Li^+ and Na^+ inhibition but as this enzyme is not essential for yeast growth it is unlikely that it

could correspond to a salt toxicity target (Lopez *et al.*, 1999). The feature in common of all these Li^+ and Na^+ inhibitory sites in phosphatases is that they correspond to weak magnesium binding sites (with affinities in the mM range) formed by phosphate groups from the substrates and carboxylate groups from the amino acids at the active site. The high charge density of Li^+ and Na^+ may displace the essential magnesium from these sites and form dead-end complexes as in inositol monophosphatase (Attack, 1995). In the case of the Hal2 enzyme, K^+ counteracts the inhibition by Na^+ and Li^+ (Murguia *et al.*, 1995) and this may be a general feature of Na^+ toxicity targets (Wyn Jones and Pollard, 1983).

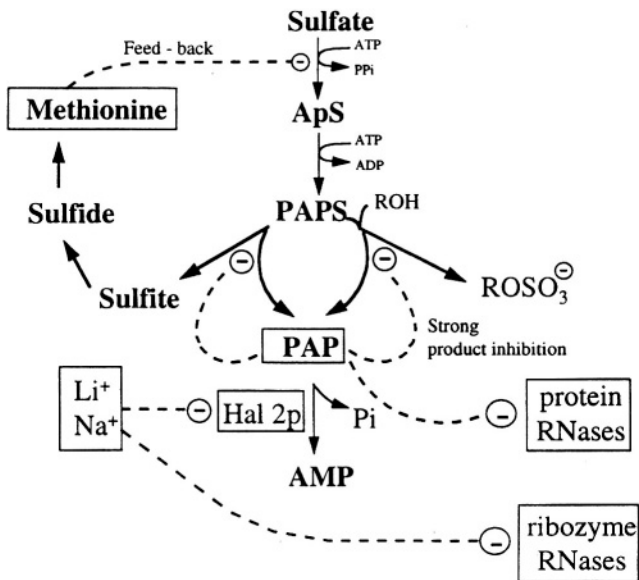


Figure 6. The mechanism of intracellular salt toxicity in yeast.

21.5 The importance of osmolyte synthesis and the elusive turgor targets

Yeast mutants deficient in glycerol synthesis are salt sensitive, highlighting the crucial role of glycerol as the osmolyte utilized for osmotic adjustment in this organism. The mutated genes include *GPD1*, encoding glycerol-3-phosphate dehydrogenase (Larsson *et al.*, 1993; Albertyn *et al.*, 1994) and the genes corresponding to components of the Hog1 MAP kinase pathway, which up-regulates *GPD1* expression in response to osmotic stress (Brewster *et al.*, 1993). It must be indicated that in wild type *S. cerevisiae* growing on glucose media the capability for glycerol synthesis is not

limiting for salt tolerance while in related yeast species with less active glycolysis, such as *Candida tropicalis*, glycerol synthesis limits salt tolerance (Garcia *et al.*, 1997).

The cellular systems injured by turgor loss during salt stress have remained elusive. From the osmosensitive phenotype of mutants it seems that both actin microfilaments (Chowdhury *et al.*, 1992) and the vacuole (Latterich and Watson, 1991) may be involved. However, the expected participation of cell wall and plasma membrane components during turgor-driven growth has not yet been disclosed by salt tolerance mutants.

21.6 What yeast mechanisms can be extrapolated to plants?

Although the basic scheme of halotolerance genes described in Figure 1 can be applied to both yeast and plant cells, it is obvious that plants are much more complicated organisms and that additional factors would play important roles in salt tolerance. For example, the regulation of xylem water flow by stomata opening and the regulation of ion inclusion in roots by transport into xylem parenchyma cells. In addition, even at the cellular level there exist important differences between yeast and plants. Plant cells subjected to osmotic stress accumulate different osmolytes than yeast cells (see Chapter 9). Although the plasma membrane and vacuolar H^+ -ATPases of yeast and plants are highly homologous, other transporters present in these membranes may be different. For example, plant plasma membranes contain inward K^+ channels (see also Chapter 18), which are not present in yeast. Also, the high affinity K^+ uptake system (probably operating as a H^+ - K^+ -symport) is mediated by different types of transporters in yeast (the Trk1,2 type) and plants (the Hak or Kup type) (Serrano *et al.*, 1999). Concerning Na^+ efflux, an Enal efflux pump is probably not present in plants, which contain antiporters of several types according to the emerging data of the Arabidopsis Genome Project (see for example the web site <http://www.mips.biochem.mpg.de/mips/athaliana>). Also, signal transduction pathways including protein kinases, protein phosphatases and transcription factors are likely to differ in details between yeast and plants.

Despite the logical differences, some basic mechanisms may be conserved. For example, K^+ uptake in yeast and plants is activated by K^+ starvation. Expression of the yeast *HAL1* gene (encoding a regulator of K^+ transport in yeast; Rios *et al.*, 1997) in transgenic melon (Bordas *et al.*, 1997) and tomato (Gisbert *et al.*, 2000) plants results in salt tolerance, leaving open a possible conservation of regulatory mechanisms. Also, Ca^{2+} and a Ca^{2+} binding protein with homology to the regulatory subunit of calcineurin, regulate K^+ transport and salt tolerance in Arabidopsis (Liu and Zhu, 1998), a mechanism similar to the one operating in yeast (Figure 5). In this respect it is relevant that expression in transgenic plants of yeast calcineurin improves salt tolerance (Pardo *et al.*, 1998), pointing again to a possible conservation of regulatory mechanisms (Chapter 18).

When yeast is compared with such evolutionary distant organisms as higher plants, a lot of caution is needed to interpret the apparent similarities in physiological mechanisms of salt tolerance. For example, the plant Hal2 homologues are probably not involved in sulfate assimilation but in sulfotransferase reactions (Gil-Mascarell *et*

al., 1999). However, as some isoforms of plant Hal2 are sodium sensitive, toxic PAP concentrations could accumulate in plants subjected to salt stress. At variance with yeast, under many circumstances the plant limiting factor during salt stress is osmotic stress, not ion toxicity. Concerning the relative toxicities of Na^+ and Cl^- , the dominant role of Na^+ in the salt tolerance of *S. cerevisiae* cannot be extrapolated to even a related yeast, *Schizosaccharomyces pombe*, where Cl^- toxicity appears to be dominant (Sugiura *et al.*, 1998). The situation with higher plants remains unknown.

Perhaps the most important lesson from the yeast work is that plant biologists should exploit a good experimental system such as *Arabidopsis thaliana* to dissect by mutational analysis the halotolerance genes of this higher plant. These halotolerance genes should include determinants of specific plant functions such as osmolyte synthesis, salt inclusion, stomata regulation etc. Later on, the basic scheme obtained with *Arabidopsis* could be compared with that obtained with yeast and adapted to other plants less amenable to a molecular genetics approach.

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CHAPTER 22

THE LONG AND WINDING ROAD TO HALOTOLERANCE GENES

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Abstract

In the past two decades a large number of genes have been shown to be associated with osmotic stress. Most of these are known only to have their expression, or more precisely, accumulation of their transcripts controlled by stress exposure. More recently, several genes have been examined in terms of the effect of their overexpression on osmotic tolerance of transgenic plants. These experiments have confirmed our understanding that tolerance is controlled by several genes, and that any single gene is unlikely to control tolerance to a major degree. Identification of several more genes that can control osmotic stress tolerance is rapidly proceeding through genomics based technologies, especially by mutant isolation and subsequent gene cloning. Eventually, microarray and bioinformatics approaches will also contribute significantly to both our understanding of osmotic stress tolerance and our ability to manipulate genetically this important trait.

22.1 Introduction

*"Water, water, everywhere,
And all the boards did shrink;
Water, water, everywhere,
Nor any drop to drink."*

from: The Rime of the Ancient Mariner

by: Samuel Taylor Coleridge

Salt, along with bread is our daily sustenance. Yet too much in our water and as Coleridge so poetically reminded us, we can die of thirst even in the midst of oceans of

water. And so it is with our crop plants as well. Undoubtedly, as soon as human civilizations began systematic agriculture, salinity and associated water deficit stresses must have become a problem for our ancestral farmers (see also Chapter 1). Food and fiber source plants were clearly chosen and domesticated for maximum productivity and not tolerance to abiotic stresses. It has been the role of farming itself to overcome the limitation set by these stresses through irrigation and other management and cultural practices (O'Leary, 1994). It is only under circumstances of global production limitations that we have begun to look back at how the lack of tolerance to the stresses appeared in the crop plant species that we now so heavily depend upon.

We can, and have, pondered a lot about how our crop species have ended up so osmotically sensitive (see O'Leary 1994). Did we systematically over time select away from tolerance to boost productivity? Were there other factors involved that precluded stress tolerance in the original choices of the elite group of archeotype crops? Virtually all of our food (95%) is produced from only 30 plant species out of the several hundred thousand known, and 50% of the food produced on our planet is from only four species, rice, maize, wheat and potato (Mann, 1999; Janick, 1999). In spite of the fact that agricultural overproduction is an economic problem for producers in many developed countries, the global human population is now faced with increasing demands for greater overall crop productivity often under conditions of salinity and associated desiccation stress. A closer look at the underlying molecular basis of osmotic tolerance/sensitivity is now clearly warranted.

Based on extensive studies on the responses of plants to abiotic stresses that have been compiled so far, we can make some important conclusions (Zhu *et al.*, 2000; Hasegawa *et al.*, 2000; Zhu *et al.*, 1997; Bohnert *et al.*, 1995). First, there is tremendous diversity within the plant kingdom for tolerance to osmotic stresses. Second, all plants have some capacity to respond to osmotic stress and become more tolerant, but they vary greatly in the speed and ultimate degree limiting their response. Third, the types of physiological and biochemical responses that have been observed in different plant species vary enormously, yet are also sometimes shared over wide ranges of phyla. Fourth, although it is clear that many genetic loci impact salinity tolerance, just a few loci can have major influences (Dubcovsky *et al.*, 1995; Zhong and Dvorak, 1995; Gorham *et al.*, 1997). Fifth, individual responses that appear closely associated with increased tolerance can be very complex or in some instances very simple. In fact, Serrano (1996) has pointed out that animals and plants appear to have diverged in the way that they control chemoosmotic gradients, animals utilizing a Na^+ pump and plants opting for a H^+ pump. This simple difference dictates to a large extent the dependence of plants on alternative and still not completely understood biochemistry to deal with steep Na^+ gradients. Since even the sensitive crop plants can adjust to small, slowly increasing Na^+ gradients (Niu *et al.*, 1995; Bressan *et al.*, 1982; Bressan *et al.*, 1981; Läuchli, 1976) it is the rapid large increases in external Na^+ that severely injures or kills these plants precluding their eventual adjustment (Bressan *et al.*, 1990; Läuchli, 1984).

If we were to take these points to heart and try to formulate a reasonable "first-phase" plan to accomplish significant genetic improvement of salinity tolerance of our important crop species, we should recognize that the most crucial adaptive response is the ability to deal with large rapid changes in the Na^+ gradient. Then we could begin to identify the specific genes responsible for this ability so that gene transfer to many crops

could be achieved. This has been the ultimate goal of molecular genetic research on stress tolerance.

22.2 Identification of stress adaptation genes

22.2.1 THE EARLY YEARS

It seems that earlier attempts by conventional breeding and genetics that generally failed to identify any simple adaptive physiological or biochemical trait led to much debate and skepticism over our ability to genetically modify the osmotic tolerance of crop plants. There was so much skepticism that for many years a mirror image approach of domesticating already osmotic tolerant halophytes, (for example *Salicornia* or *Atriplex*) or xerophytes (for example cacti) was a serious business - and still remains an interesting and useful approach (O'Leary, 1994; Glenn *et al.*, 1999). Yet powerful social and economic forces resist the introduction of new crops (Janick, 1999) so that the improvement of stress tolerance of our elite crop species is imperative. Perhaps in response to the skepticism over the existence of any "important simple" tolerance trait, but more likely because of the basic do-ability of hunting for stress-induced genes or gene products, these searches dominated our whole mind-set in our attempts to understand the molecular genetic basis of stress adaptation in the last two decades. What follows is a summary of the somewhat disappointing history of this pursuit and finally an optimistic view of the rapidly changing landscape of stress tolerance gene discovery.

22.2.2 THE PROTEIN YEARS

Numerous studies resulted in the identification of changes in levels of accumulation of several proteins in response to osmotic stress (Hurkman and Tanaka, 1987; Hurkman and Tanaka, 1988; Hurkman *et al.*, 1994; LaRosa *et al.*, 1989; Singh *et al.*, 1985; Reid and Walker-Simmons, 1993). Usually these proteins were reported only as visible changes in protein patterns on acrylamide gels. However, many of the altered bands or spots have been identified (Dure III, 1993; Handa *et al.*, 1986; Vierling, 1991; Zhu *et al.*, 1993; Zhu *et al.*, 1994). Unfortunately, the fact that an altered level of accumulation in and of itself does not prove the function of a protein, and the incomplete nature of such screens (many proteins are not abundant enough to visualize on these gels), did not leave us with a very complete nor accurate view of the involvement of induced proteins in the manifestation of a tolerance phenotype (Zhu *et al.*, 1997). So, this sort of "land-rush" for stress proteins which really began in earnest in the early 1980s with various limited sorts of induced/uninduced comparisons of protein profiles from sensitive/less sensitive species, lines, cultivars, etc., and culminated with some pretty impressive 2-D gel comparisons, left us with only a large, interesting but not much more than a shopping list of altered bands or spots.

22.2.3 THE RNA YEARS

This race to find the important genes controlling stress tolerance intensified in the mid 1980's with a panoply of new techniques to identify gene transcripts that accumulate in

response to osmotic or related stresses such as cold. These screens for induced transcripts were much more extensive and sensitive than the forerunner attempts to identify induced proteins directly. In addition, DNA sequencing which is much more feasible than protein sequencing allowed the sequence identification of many more of these stress-induced transcripts. As a consequence, some general patterns or categories of genes that are responsive to osmotic stress did emerge from this work. Only some examples from these general categories are given here. We have not attempted to make this list complete. It serves no compelling purpose to do this, since the list is still far from finished and our only clear conclusion now is that the number of stress-induced genes is large. As we shall discuss later, the emerging technology of microarray analysis will eventually provide us with the complete list of stress-activated genes.

22.3 Stress induced genes

22.3.1 GENES ENCODING TRANSPORTER PROTEINS

Several genes encoding proteins involved in the transport of various ions and molecules have been identified (Mathuis and Sanders, 1992; Schroeder *et al.*, 1994). Several ATPase encoding genes are induced osmotically including both plasma membrane and tonoplast types (Niu *et al.*, 1993a; Niu *et al.*, 1993b; Surowy and Boyer, 1991; Sze 1985; Perez-Prat *et al.*, 1994; Wimmers *et al.*, 1992; Braun *et al.*, 1986; Narasimhan *et al.*, 1991; Perez-Prat *et al.*, 1992; Sze, 1985; Wada *et al.*, 1992; Binzel, 1995). Many genes encoding proteins thought to be involved in water transport have now been recognized as osmotically inducible (Yamada *et al.*, 1995; Yamaguchi-Shinozaki *et al.*, 1992).

22.3.2 GENES INVOLVED IN PHOTOSYNTHESIS

Many genes involved in the switch from C3 type photosynthesis to CAM photosynthetic metabolism have been identified and intensively studied (Cushman *et al.*, 1989; Cushman *et al.*, 1990; Ostrem *et al.*, 1990; Locy *et al.*, 1996; Winicov, 1994; Chapter 17). The roles of these genes in adaptation to stress are probably related to long-term adaptive responses rather than initial survival responses. This subject has been reviewed in detail by Cushman and Bohnert (Chapter 17).

22.3.3 LEA GENES

The largest and most complex group of genes induced by osmotic stress are the LEA genes (Almoguera and Jordano, 1992; Baker *et al.*, 1988; Dure, III *et al.*, 1989; Reid and Walker-Simmons, 1993; King *et al.*, 1993; Hong *et al.*, 1988; Mundy and Chua, 1988; Pla *et al.*, 1989; Piatkowski *et al.*, 1990; Dure III, 1993; Close *et al.*, 1988; Skriver and Mundy, 1990). This name refers to **L**ate **E**mbyrogenesis **A**bundant proteins/genes that were identified originally as being induced during the desiccation stage of seed development. This important family of genes has been intensively studied and many attempts to ascertain their function during osmotic stress have been made. Unfortunately, no clear answer to the question of their function has emerged although it

is likely that they play roles in several mechanisms of protection including membrane stabilization, retention of water around sensitive cellular structures and compartments, and chaperone-like stabilizations (Dure III, 1993). As we shall see later, LEA genes have served as genes with important stress-controlled promoters used to identify transcription factors.

22.3.4 RED HERRING GENES

Genes encoding PR-5 and other pathogenesis related protein families are strongly induced by osmotic stress (Pierpoint *et al.*, 1981; Ohashi and Matsuoka, 1985; Yun *et al.*, 1996, 1997). An abundance of work by several investigators has demonstrated a clear anti-microbial function for these proteins (Broglie *et al.*, 1991; Alexander *et al.*, 1993; Liu *et al.*, 1994; Ponstein *et al.*, 1994; Woloshuk *et al.*, 1991). It is important to mention this group of osmotically induced genes because they represent a clear example of genes that are osmotically induced but have no apparent function that is involved with tolerance to osmotic stress. This disconnection between the differential expression of a gene and its function is now clearly recognized as a general phenomenon in gene expression.

The reason for the induction of genes whose function is unrelated to the conditions that induce their expression is not entirely understood. However, it has become an accepted view that this induction is mechanistically the result of a very complex redundant and overlapping signal perception system (Shinozaki and Yamaguchi-Shinozaki, 1996).

22.3.5 VARIOUS OTHER OSMOTICALLY-INDUCED GENES

Through the use of various techniques, a number of other genes have been found to be stimulated by exposure to osmotic stresses. Water channel proteins that are thought to involve the control a water movement through membranes are specifically controlled by osmotic stress in various plant tissues (Chrispeels and Agre, 1994; Yamada *et al.*, 1995; Jones and Mullet, 1995; Bohnert *et al.*, 1995; Bartels and Nelson, 1994). Proteins thought to have some protective function for membranes or other cellular structures such as lipid desaturase (Bray, 1993; Thomashow, 1994; Bohnert *et al.*, 1995; Bartels and Nelson, 1994) lipid transfer proteins (Torres-Schumann *et al.*, 1992) and proteins involved in stabilizing proteins or mRNA (Bohnert *et al.*, 1995; Bartels and Nelson, 1994; Goday *et al.*, 1994; Kiyosue *et al.*, 1994) are also stimulated during stress. Finally, a number of genes encoding proteins involved in protein turnover and detoxification including oxalate oxidases (Membre *et al.*, 1997; Michalowski and Bohnert, 1992; Hurkman *et al.*, 1994; Hurkman and Tanaka, 1996), glutathione S-transferase, catalase, ascorbate peroxidase, peroxidase and epoxide hydrolases are activated also (Lucas *et al.*, 1996; Gupta *et al.*, 1993; Bray, 1997; Bohnert *et al.*, 1995; Bartels and Nelson, 1994; Prasad *et al.*, 1994; Mittler and Zilinskas, 1994; Kalir *et al.*, 1981; Sachs and Ho, 1986; Kiyosue *et al.*, 1994).

22.3.6 GENES CONTROLLING METABOLIC PATHWAYS THOUGHT TO PARTICIPATE IN OSMOTIC STRESS TOLERANCE

Even before investigators began to identify protein changes in plants exposed to osmotic stress, a number of metabolites were found to accumulate in response to stress exposure (Yancey, 1994). Prominent among these metabolites is the amino acid proline and quaternary ammonium compound betaine. These and other metabolites have long been considered to accumulate as compatible osmolytes that are sequestered in the cytoplasm to counterbalance ions that are compartmentalized in the vacuole (Niu *et al.*, 1995; Rhodes and Samaras, 1994; Chapter 9). Considerable effort has been expended to determine the enzymes that limit or control the accumulation of these osmolytes. All of the genes encoding the enzymes involved in the synthesis of proline and betaine have now been isolated and characterized (Delauney and Verma, 1990; García-Ríos *et al.*, 1997; Maggio *et al.*, 1996; McCue and Hanson, 1991; Weretilnyk and Hanson, 1990). The enzyme responsible for proline catabolism has also been cloned (Kiyosue *et al.*, 1996; Verbruggen *et al.*, 1996). Betaine is not broken down by plants but is simply diluted during growth. A number of other genes encoding enzymes involved in the metabolism of so-called osmolytes have been isolated and several of these have been used to produce transgenic plants that overaccumulate these solutes (Bohnert and Shen, 1999). These engineering efforts represent the first wave of genetic manipulations that were intended primarily to determine the role of osmolytes in adaptation, but also to produce osmotic stress tolerance plants.

22.4 Osmotic stress tolerance: the first wave of transgenic plants

Overexpression of stress related genes in transgenic plants has been a common approach toward the elucidation of the molecular and physiological basis for salt tolerance, and an obvious “next step” after the molecular analysis of gene expression patterns in osmotically stressed plants. Despite the well-known limitations of a “single-gene” strategy, as a way to improve plant performances in saline environments (Zhong and Dvorak, 1995), the genetic engineering of plants with single genes to allow overaccumulation of end-products of biochemical pathways has been conceptually too simple and too tempting to be overlooked, and numerous reports of these experiments have appeared in the past several years (*Table 1*). The picture that has emerged from these studies is far from clear, but does indicate that this strategy can lead only to marginal increments in stress tolerance, often limited to specific laboratory conditions or narrow time or developmental windows of response. In none of these studies lies the degree to which metabolites have accumulated in the transgenic plants been sufficient to account for significant adjustment of cellular osmotic potential (Morgan, 1984). This has led to the speculation that overaccumulation of many solutes such as mannitol, proline, etc. protects cells from injury resulting from NaCl-induced reactive oxygen species (ROS) (Bohnert and Shen, 1999). Indeed, transgenic plants expressing enzymes involved in detoxification of ROS have been shown to be more NaCl-tolerant (Bowler *et al.*, 1991; Roxas *et al.*, 1997; Shen *et al.*, 1997a,b; Mc Kersie *et al.*, 1996; Gupta *et al.*, 1993). An important possibility generally underestimated in many of these studies is that the transgene can affect the growth of the plant in the absence of stress. Most of the

studies dealing with gene overexpression to improve osmotic stress tolerance provide marginal or insufficient information on plant growth rates. Therefore, it has been difficult (or impossible) to assess objectively the potential physiological and agronomic significance of the altered phenotypes that were observed. Overexpression of the *Mt1D* gene from *Escherichia coli* and *TPS1* from *Saccharomyces cerevisiae* in transgenic tobacco, encoding a mannitol 1-P-dehydrogenase and a trehalose-6-phosphate synthase respectively, are the only two examples where the effect of the transgene on plant growth rates has been clearly assessed (Karakas *et al.*, 1997; Romero *et al.*, 1997). In both cases, growth was clearly impaired in the transgenic plants regardless of the presence or absence of stress. It can be argued, therefore, that the tolerance observed in presence of high salt (*Mt1D*-transgenic plants) or water stress (*TPS1*-transgenic plants) could be linked to phenomena that are actually caused by reduced growth (i.e. reduced Na^+ uptake in presence of NaCl-induced stress or reduced water consumption) (Dalton *et al.*, 2000; 2001; Karakas *et al.*, 1997; Dalton *et al.*, 1997; Bressan *et al.*, 1982). Although the linkage between reduced growth and osmotolerance is not a new concept (Bressan *et al.*, 1982; 1990), transgene-induced growth inhibition could be interpreted in two different ways: as a simple unexplained artifact or as part of a complex adaptive response triggered by some signaling event (such as the accumulation of solute molecules) that also affects the control of cell growth. Another largely overlooked possibility in many of these studies is the likelihood that the accumulation of various solutes in transgenic plants could reflect much more dramatic changes in flux rates of particular metabolites. This could lead to alterations in related metabolism or even the generation of a metabolic futile cycle (Hare and Cress, 1997) and thus a limitation in a metabolite essential for growth such as ATP, NADPH etc.

A. Maggio *et al.* (unpublished results) have recently expressed in *S. cerevisiae* *tomPRO2*, a P5CS from tomato, and mutated derivatives of *tomPRO2*. Six different strains were obtained carrying the structural yeast P5CS, the wild type plant gene (*tomPRO2*) and two different mutated derivatives (*tomPRO2-13* and *tomPRO2-20*) alone and in combinations with knock-outs of the structural gene encoding proline oxidase (catalyzing the first step of proline catabolism), respectively. These strains accumulated different levels of proline and displayed a strong inverse relationship between internal proline concentration and growth rate. Taken together with the reports of reduced growth in transgenic plants, these observations have produced a growing suspicion that growth rate and salt stress tolerance are probably coupled and coordinated events possibly linked by the level of cellular solutes (Bressan *et al.*, 1990; Maggio *et al.*, 1997).

22.5 Perception of stress and signal transduction

This brings us to our current state of understanding concerning the importance of particular genes in controlling stress tolerance. In order to mount an effective defense against a hostile osmotic environment, plants need to perceive the presence of such an environment and effectively activate the genes needed to bring about all appropriate changes in physiology, biochemistry, morphology, and ontology involved in a successful survival strategy. This means that all genes involved in tolerance can be divided into two conceptually important categories. 1) Genes encoding proteins

TABLE 1. Transgenic plants engineered for tolerance to salinity stress, water deficit or oxidative stress.

Plant species	Transgene (origin)	Function of the transgene	Effect of the transgene on plant growth, in the absence of stress	Reference
OSMOLYTES				
<i>Nicotiana tabacum</i>	<i>MtID</i> (<i>E. coli</i>)	enhances mannitol accumulation	Reduced growth	Tarczynski <i>et al.</i> , 1992, 1993;
<i>N. tabacum</i>	<i>P5CS</i> (<i>Vigna aconitifolia</i>)	enhances proline accumulation	No growth reduction	Karakas <i>et al.</i> , 1997
<i>Arabidopsis thaliana</i>	<i>MtID</i> (<i>E. coli</i>)	enhances mannitol accumulation	ND	Kishor <i>et al.</i> , 1995
<i>N. tabacum</i>	<i>SaeB</i> (<i>B. subtilis</i>)	enhances fructan accumulation	No growth reduction	Thomas <i>et al.</i> , 1995
<i>N. tabacum</i>	<i>TPS1</i> (<i>S. cerevisiae</i>)	enhances trehalose accumulation	Reduced growth	Pilon-Smits <i>et al.</i> , 1995
<i>N. tabacum</i>	<i>Imt1</i> (<i>M. crystallinum</i>)	enhances D-ononitol accumulation	ND	Holmström <i>et al.</i> , 1996; Romero <i>et al.</i> , 1999
<i>A. thaliana</i>	<i>CodA</i> (<i>Arthrobacter globiformis</i>)	enhances glycinebetaine accumulation	ND	Sheveleva <i>et al.</i> , 1997
<i>Oryza sativa</i>	<i>P5CS</i> (<i>V. aconitifolia</i>)	enhances proline accumulation	ND	Hayashi <i>et al.</i> , 1997
				Zhu <i>et al.</i> , 1998
SIGNAL TRANSDUCTION				
<i>N. tabacum</i>	<i>CaV</i> (<i>S. cerevisiae</i>)	regulator of ion homeostasis (yeast)	ND	Pardo <i>et al.</i> , 1998
<i>A. thaliana</i>	<i>DREB1A</i> (<i>A. thaliana</i>)	transcription factor	Reduced growth ⁽²⁾	Liu <i>et al.</i> , 1998; Kasuga <i>et al.</i> , 1999
<i>Curcubita melo</i>	<i>HAL1</i> (<i>S. cerevisiae</i>)	regulator of ion homeostasis (yeast)	ND	Serrano <i>et al.</i> , 1999
<i>N. tabacum</i>	<i>At-DBF2</i> (<i>A. thaliana</i>)	signal molecule	ND	Lee <i>et al.</i> , 1999
<i>Medicago sativa</i>	<i>Alfin1</i> (<i>M. sativa</i>)	transcription factor	ND	Winicov <i>et al.</i> , 1999
PROTECTION V/S. ROS⁽¹⁾				
<i>N. tabacum</i>	<i>MnSOD</i> (<i>N. plumbaginifolia</i>)	free radicals scavenger	ND	Bowler <i>et al.</i> , 1991
<i>M. sativa</i>	<i>MnSOD</i> (<i>N. plumbaginifolia</i>)	free radicals scavenger	ND	McKersie <i>et al.</i> , 1993, 1996
<i>N. tabacum</i>	<i>FeSOD</i> (<i>A. thaliana</i>)	free radicals scavenger	ND	Van Camp <i>et al.</i> , 1996
<i>N. tabacum</i>	<i>MtID</i> (<i>E. coli</i>)	free radicals scavenger	ND	Shen <i>et al.</i> , 1997a,b
<i>N. tabacum</i>	<i>Gst/Gpx</i> (<i>N. tabacum</i>)	free radicals scavenger	ND	Roxas <i>et al.</i> , 1997
Ion homeostasis				
<i>A. thaliana</i>	<i>ANH1</i> (<i>A. thaliana</i>)	Na ⁺ /H ⁺ antiporter	ND	Apse <i>et al.</i> , 1999
Others				
<i>O. sativa</i>	<i>Hva1</i> (<i>Hordeum vulgare</i>)	unknown (LEA protein)	ND	Xu <i>et al.</i> , 1996

⁽¹⁾ Reactive oxygen species⁽²⁾ 3S:DREB1A inhibits growth; RD29A:DREB1A does not inhibit growth.

involved in the perception and signaling that a change in the osmotic environment has occurred and 2) genes that respond to these signals and are responsible for "redesigning" the plant so that it survives and reproduces in the new environment. Most of the stress-associated genes that have been identified so far fall into the second category including all those just summarized as being introduced into transgenic plants. It has become increasingly clear, however, that genes in the first category that are involved in perception and signal transduction are potentially far more useful in future attempts to genetically modify the stress tolerance capabilities of important crop plants because they control the expression of a large number of genes in the second category (Pardo *et al.*, 1998; Serrano *et al.*, 1999).

Since genes involved in perception and signaling need to be expressed at all times in order that the perception and signaling mechanism be in place to allow detection of any change, approaches that utilize differential expression techniques would be unlikely to uncover signaling components. Yet in systems where signaling has been extensively studied (such as mating response in yeast) some of the genes encoding signaling proteins can be further induced after exposure of the cells to the environmental cues connected to the signal (mating) pathway (Hall *et al.*, 1996). This stimulation of gene expression could function as a particular type of amplification of the signal response. In fact, a number of plant genes have been identified by screens designed to detect gene-induction by stress that appear to encode signal transduction proteins. They have been identified as signal components by virtue of their sequence similarity to genes known to encode signal proteins such as kinases and phosphatases (Mizoguchi *et al.*, 1996; Chang *et al.*, 1993). As we shall see later, this ability to surmise function of a gene by virtue of its sequence similarity to other genes forms the basis of bioinformatics.

22.6 How can genes that function in signal pathways be identified?

Once it became clear that there were large numbers of genes that are activated by osmotic stress, most investigators sensed that strategies to get at the regulatory genes would be needed. The most common first approach to uncover regulatory genes involved the use of promoter elements of genes that are strongly induced by a stress environment. These specific sequences, further in the 5' direction from the amino acid coding region of stress-stimulated genes, are responsible for the stress inducibility because they interact with other elements of the stress perception system by physically binding to one or more of those components referred to as transcription factors (Oeda *et al.*, 1991; Gultinan *et al.*, 1990; Marcotte *et al.*, 1992; Marcotte *et al.*, 1989). A number of transcription factors involved in activation of stress responsive genes have been identified by using these promoter elements as specific probes (Yamaguchi-Shinozaki and Shinozaki, 1994; Shen and Ho, 1995; Xu *et al.*, 1998). The most intensively studied example of such transcription factors was found to encode a protein that controlled expression of both cold-induced and osmotically-induced genes. DREB (Liu *et al.*, 1998) and CBF (Stockinger *et al.*, 1997) are transcription factors that were discovered as activators of the osmotically induced *RD29* gene and of the cold-induced *COR-78* gene respectively. It turns out that the *DREB* and *CBP* genes encode essentially the same protein that interacts with a DRE (Dessication Responsive Element) that also responds to NaCl stress on the *RD29* gene (Figure 1). The *RD29* gene also contains

separate sequences responsible for its responsiveness to ABA, the ABRE (ABA Responsive Element) (Fig 1). Detailed analyses of the responsiveness of this gene and others has led to the prediction of at least 5 pathways both ABA dependent and independent, that control adaptation to osmotic stress (Shinozaki and Yamaguchi-Shinozaki, 1996).

When the *DREB/CBP* genes were transformed into *Arabidopsis* under the control of a constitutive promoter, many downstream stress-induced genes became constitutively activated (Jaglo-Ottosen *et al.*, 1998). These transformed plants also tended to validate the developing strategy that activating a signal component would result in better stress tolerance than had been observed with previous gene transfer experiments. The increase in stress tolerance of plants ectopically expressing these transcription factors did exhibit generally greater levels of stress tolerance than the levels observed in plants engineered to activate a specific response such as mannitol or trehalose overproduction etc. (Tarczynski *et al.*, 1992; Holmström *et al.*, 1996) (see Table 1). Nevertheless, levels of

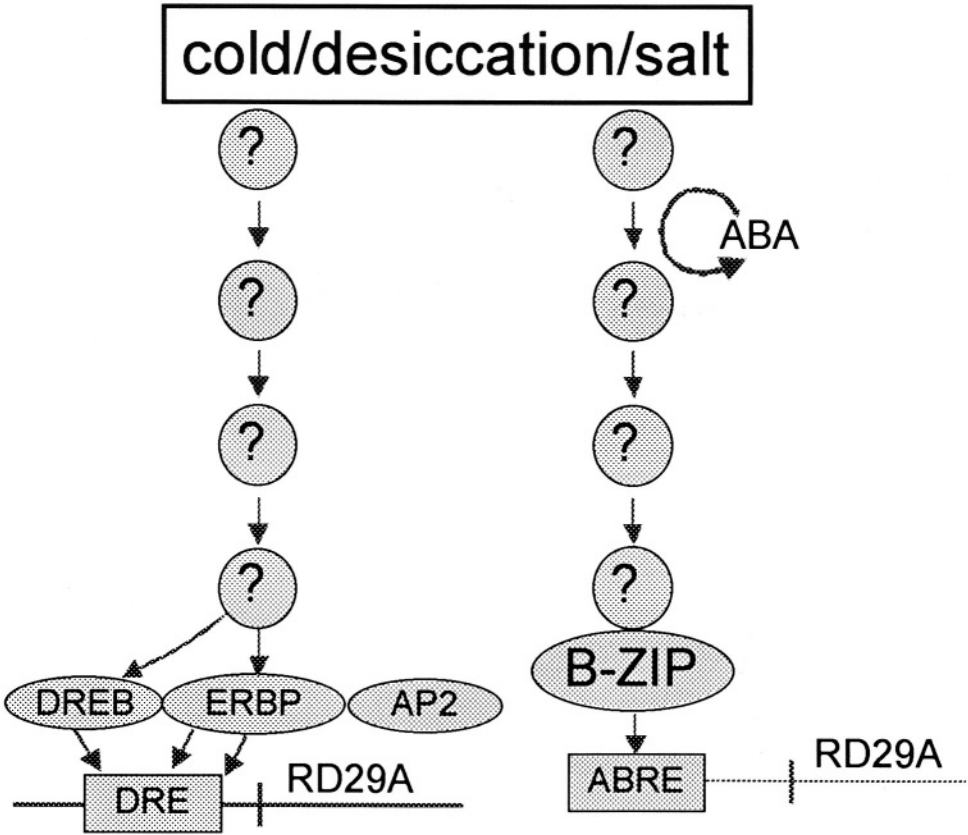


Figure 1. Examples of ABA dependent and ABA independent induction of genes by osmotic signalling pathways that lead to different promoter elements and control expression of the *RD29* LEA gene.

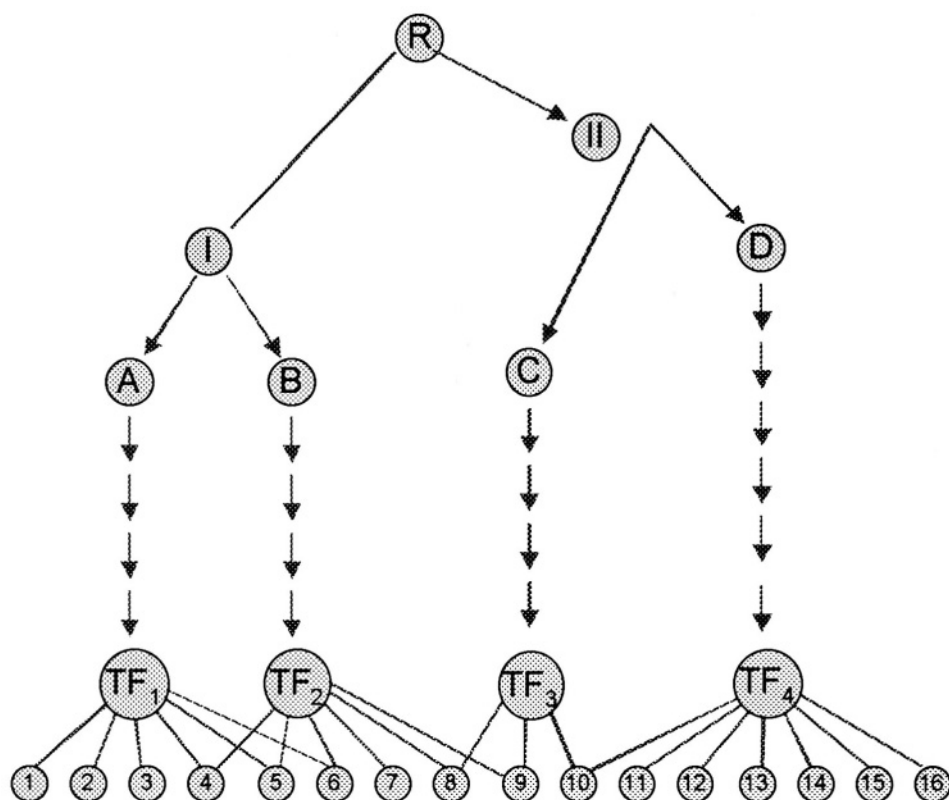


Figure 2. Complexity of signal pathways leading from salt stress receptor(s) to arrays of activated genes (1-16) indicates that receptor genes or genes encoding components near the receptor will be needed to activate larger arrays of genes and impact the adaptive response more effectively. R or I and II or A, B, D or TF₁₋₄ are needed to activate all arrayed genes. B and D activate 81% of arrayed genes.

stress tolerance observed in these experiments with transcription factors were still not dramatic enough to be considered useful in field situations. An important consideration in this regard is the fact that transcription factors are at the end of signal cascades and are probably responsible for activation of the smallest subsets of downstream genes (see *Figure 2*). The strategy to isolate signal components could now be considered on the right trail, but genes encoding signal proteins closer to the point of stress perception would likely be needed for better activation of larger arrays of genes responsible for phenotype conversion to a tolerant state.

A major fundamental drawback to the approach of identification of induced genes and subsequently the transcription factors that control their induction is that this process does not actually utilize any test of function for the gene identification process itself. Rather, it relies on the assumption that the induced gene (and its promoter) lies at the end of a pathway that actually controls a significant part of the tolerance response. Keep

in mind that several genes that are osmotically induced apparently do not function in osmotic tolerance at all (see section 2.4 on Red Herring genes).

22.7 The use of model systems to reveal genes that function in stress tolerance

Although it was becoming clearer that the best tolerance genes would most likely be those involved at or near the point of perception of an osmotically stressful environment because these genes control the expression of more downstream genes (*Figure 2*), previous strategies were not very useful to get at these genes. The fact that the best approach to identify these genes would require the ability to examine changes quickly in a tolerance phenotype began to be recognized. This recognition has driven this type of research toward the use of model systems that allow rapid detection of phenotype changes resulting from gene mutations.

22.8 Rapid advances in model systems are shortening the time between mutant phenotype discovery and gene identification

Once an individual mutant with either increased or decreased tolerance to osmotic stress has been identified, the gene responsible for the phenotype change must be isolated if our goal to determine the important genes controlling the tolerance phenotype is to be achieved. Although several organisms have been used in attempts to do this, such as *Chlamydomonas* (Prieto *et al.*, 1996; Pardo *et al.*, 1995), *Ceratopteris* (Vogelien *et al.*, 1993) and *Synechocystis* (Jeanjean *et al.*, 1990; Hagennan and Zuther, 1992), the yeast model has surfaced as the most important and successful system for this approach (Serrano, 1996; Chapter 21). Laboratory strains of the yeast *Saccharomyces cerevisiae* are haploid with a diploid phase during sexual reproduction. Yeast is easily grown under defined conditions and large numbers of variant phenotypes can be selected or screened rapidly. The entire genome is sequenced and well organized in databases. Efficient gene replacement and tagging mutagenesis systems have been developed and are extensively used already. These features provide an immensely powerful advantage in searching for stress tolerance genes. For instance, by using Tn-tagged mutant libraries the gene responsible for a mutant phenotype can be recovered and its sequence determined in a few weeks.

22.9 Osmotic tolerance genes that have been identified using the yeast model system

Compared to plants, our knowledge of the genes involved in osmotic adaptation in yeast is substantial. The lab of Ramon Serrano has extensively focused on the identification of halotolerance (HAL) determinants that mediate functional salt tolerance sufficiency in wild type yeast cells (Gaxiola *et al.*, 1992; Murguia *et al.*, 1996; Ferrando *et al.*, 1995). Here we present only a brief summary of the genetic factors known to control salt tolerance in yeast and we refer to Chapter 21 by Serrano for a more detailed discussion on this subject. Yeast is the only system where we know an entire signal pathway

leading from perception of osmotic stress to activation of downstream genes by transcription factors (Brewster *et al.*, 1993; Maeda *et al.*, 1994). This signal system is the High Osmolarity Glycerol (HOG) pathway that controls the synthesis and retention of intracellular glycerol allowing rapid and substantial osmotic adjustment in response to osmotic upshock. Another well understood osmotic signal cascade in yeast is the so-called calcineurin (CaN) pathway where this Ca^{2+} /calmodulin-dependent PP2B phosphatase is a pivotal intermediate. Although the stress signal perception component of this pathway has yet to be identified, it is activated by Na^+ and regulates transport systems responsible for ion homeostasis.

Several plant genes have been isolated that are capable of functioning to suppress a salt sensitivity phenotype of yeast cells bearing mutations in components of these signal pathways. Here we summarize these and other plant genes able to affect salinity tolerance in yeast.

22.9.1 PLANT GENES THAT SUPPRESS HOG PATHWAY COMPONENTS

A putative MAP kinase from *Pisum sativum* (PsMAPK) has been shown to have 47% primary sequence identity to yeast HOG1. PsMAPK functionally suppressed *hog1* cell growth inhibition in high salinity (Pöpping *et al.*, 1996). Two *Arabidopsis thaliana* proteins, ATMAPK4 (MAPK) and ATMEKK1 (MAPKKK) interacted specifically with the *A. thaliana* MAPKK, MEK1, in the yeast 2-hybrid protein-protein analyses (Mizoguchi, *et al.*, 1998). Further, combinations of ATMAPK4 and MEK1, and ATMEKK1 and MEK1 could suppress growth defects of yeast mutants (*mpk1Δ* and *pbs2Δ*, respectively) implicating functional activity as components of a MAP kinase cascade. Together, these results identify interacting components of a MAP kinase pathway in *Arabidopsis thaliana*.

22.9.2 PLANT GENES THAT SUPPRESS CALCINEURIN (CAN) PATHWAY COMPONENTS

Two *Arabidopsis thaliana* genes were isolated as functional suppressors of CaN deficient mutants (*cna1* and *cna2* or *cnb1*). The gene products, STZ and STO, have sequence similarity to zinc finger type transcription factors (Lippuner *et al.*, 1996). Suppression of salt sensitivity by STZ was dependent on ENA1 whereas STO acted independently of the Na^+ -ATPase. Recently, a novel tobacco suppressor of a CaN deficient mutant has been isolated that mediates salt tolerance sufficiency through a mechanism that involves *ENA1* activation (Matsumoto, 1999). The ortholog from *A. thaliana* also suppressed the salt sensitive phenotype of a CaN deficient mutant.

22.9.3 PLANT GENES RELATED TO OTHER YEAST DETERMINANTS OF SALT TOLERANCE

A. thaliana *SAL1* was isolated by complementation of a yeast *ena1* mutant (Quintero *et al.*, 1996). Both *SAL1* and the rice homologue, *RHL1* (Peng and Verma, 1995), complemented the *hal2/met22*, a mutant defective in sulfate assimilation. The encoded proteins exhibited both 3'(2'),5'-bisphosphate nucleotidase (as does HAL2/MET2) and inositol polyphosphate I phosphatase activities. *SAL1* increased Na^+ and Li^+ tolerance

of yeast cells by attenuating Li^+ inhibition of the 3'(2'),5'-bisphosphate nucleotidase activity of *SAL1/HAL2* (Murguia *et al.*, 1996), and by enhancing Na^+/Li^+ efflux through activation of *ENA1* by a mechanism that was predicted to involve inositol phosphate signaling. A functional homologue of the yeast DBF2 serine/threonine kinase, was isolated in *Arabidopsis*, also (Lee *et al.*, 1999). Overexpression of *At-DBF2* increased tolerance to osmotic, salt and cold stresses in yeast and plants as well.

22.9.4 YEAST GENES CAN AFFECT PLANT SALT TOLERANCE

Considerable effort has been made to identify the plant version of the yeast calcineurin gene because it is so pivotal in controlling ion homeostasis. Although the plant calcineurin gene has not yet been discovered, the yeast calcineurin A/B genes were introduced into transgenic tobacco in an activated form resulting in increased salt tolerance (Pardo *et al.*, 1998). This tolerance was again comparable to the level seen with transcription factor transformants. Even though calcineurin is upstream of transcription factors, a high degree of tolerance could not be achieved, perhaps as a result of incompatibility of the yeast calcineurin proteins with other elements in the plant signal pathway (Pardo *et al.*, 1998). The mechanism by which the yeast calcineurin induces salinity tolerance in plants is not known although, as previously indicated it regulates multiple aspects of ion flux in yeast cells partly through stimulating transcription of target genes.

22.10 The emergence of *Arabidopsis* as the premier plant version of the yeast model system

The fact-of-life drawback of the yeast model is simply that although it produces excellent information concerning the function of genes, yeast is not a plant and any tolerance genes found in yeast need to be rediscovered or retested in plants. So, even when plant genes are found to complement or suppress osmotically sensitive mutants of yeast they need to be retested in plants for their ability to cause sensitivity when mutated or cause tolerance when overexpressed. Also, when a yeast gene is found that is capable of affecting osmotic tolerance of yeast or even of a transgenic plant, such as the case with the yeast calcineurin genes (Pardo *et al.*, 1998), the plant version of this gene needs to be identified. Therefore, the ideal system would be a plant with all of the molecular/genetic advantages of yeast. Fortunately, *Arabidopsis thaliana* is rapidly becoming such a model. Although it is not haploid and homologous recombination for use in gene replacement is poorly developed, it has been a highly successful system for the isolation of mutants (Saleki *et al.*, 1993; Feldmann *et al.*, 1991; Werner and Finkelstein, 1995). Several salt sensitive mutant plants referred to as Salt Overly Sensitive (SOS) mutants have been isolated (Wu *et al.*, 1996; Liu and Zhu, 1997) by J.K. Zhu and co-workers. One of these genes (*SOS3*) was recently identified by map-based cloning (Liu and Zhu, 1998) a technique that has been rapidly improving in *Arabidopsis* as the sequence of the genome becomes more complete. *SOS3* encodes a Ca^{2+} binding protein with sequence similarity to calcineurin B (Liu and Zhu, 1998) and may represent the plant counterpart of yeast calcineurin B (Bressan *et al.* 1998). It will be very interesting to know what downstream genes are controlled by *SOS3* and what

other signal transduction components directly interact with *SOS3* so that this pathway in plants can be compared with the yeast counterpart.

The isolation of genes involved in mutant phenotypes of *Arabidopsis* is being greatly facilitated by the use of effective T-DNA insertion mutagenesis systems utilizing an "activation" tag. These systems are rapidly proving useful to generate libraries of mutant *Arabidopsis* plants that contain both "activated" and disrupted genes (Walden *et al.*, 1994). "Activated" genes appear in the first generation as dominant mutations because the inserted DNA contains promoter/activator sequences (usually tandem repeats of the 35S promoter) that upon insertion into the genome adjacent to a transcription start of an open reading frame (ORF) initiate ectopic expression of the adjacent ORF, potentially causing an altered phenotype. These activated genes as well as disrupted genes (when the T-DNA inserts into the amino acid coding region of an ORF) can be rapidly cloned via a PCR mediated rescue technique that produces the T-DNA and its flanking region on a plasmid (usually Bluescript) that can be replicated easily in *E. coli* and sequenced. These *Arabidopsis*-based technologies are dramatically increasing the speed by which genes encoding signaling components of the stress tolerance machinery are identified.

There are other extremely important capabilities that are afforded investigators by the use of *Arabidopsis* as a model system. These capabilities will also prove invaluable in future efforts to identify the key important genes controlling adaptation to osmotic and other stresses.

22.10.1 SIGNAL TRANSDUCTION TRAPS

The fusion of osmotically-induced promoters like the *RD29* promoter to highly sensitive and easily screenable marker genes such as the *LUC* reporter gene is allowing investigators rapidly to identify genes that control expression of such promoters (Figure 3). Ishitani *et al.* (1997; 1998) have used the *LUC* gene fused to the *RD29* promoter to trap mutants with altered signal capabilities. They have classified more than 800 of these mutants into categories designated *cos* (constitutive expression of osmotically responsive genes), *hos* (high expression of osmotically responsive genes), *los* (low expression of osmotically responsive genes). The large number of mutants found by Ishitani *et al.* (1997) indicates the complexity of the osmotic signaling system in plants. Identification of the mutated genes causing the altered signaling responses can be accomplished presently only by map-based cloning procedures which still require considerable time and effort. However, these trapping systems when combined with T-DNA insertion mutation populations should allow the relatively quick identification of the genes controlling not only the downstream *RD29* gene but eventually the other genes under the control of the family of promoters to which the *RD29* promoter belongs. The use of other promoters with different spectra of downstream target genes will allow eventually the mapping of the entire osmotic signal complex.

22.10.2 REVERSE GENETIC SCREENS FOR GENE KNOCK-OUT MUTANTS

Populations of T-DNA insertion mutagenized *Arabidopsis* plants are usually kept in small family pools of 10 to 20 mutant plants per pool. This allows the rapid identification of plants harboring mutations in any gene for which the DNA sequence is

sensitivity of PCR, genomic DNA can be extracted from many mutagenized plants and combined (usually pools of 1000 plants) followed by screening with this PCR procedure. If a T-DNA insertion mutant is present among the 1000 pooled plants, the PCR reaction will generate an amplified band of DNA. Pools of 100 plants comprising any of the 1000 plant pools that tested positive are then screened, followed by screening of the 10 or 20 plant pools comprising any positive 100 plant pools. Individual plants from these smaller pools are then screened by PCR to identify the actual mutant plant. This procedure will prove extremely valuable to test the effect on stress tolerance of various genes identified by other approaches. For instance the many signal transduction genes, such as Mitogen Activated Protein, MAP-kinases (Mizoguchi *et al.*, 1993, 1996; Jonak *et al.*, 1996); Calcium Dependent Protein Kinases, CDPKs (Urao *et al.*, 1994) phospholipase C (Hiyayama *et al.*, 1995) and transcription factors such as MYB (Urao *et al.*, 1993) that were identified as being induced by stress can be tested this way individually and in combinatorial knock-outs by crossing single gene knock-out mutants with each other and screening for double knock-outs. In addition, other genes suspected to be involved in the direct manifestation of the tolerance phenotype such as K^+ transport proteins that could represent potential competition sites by which Na^+ ions may enter the cell (Schroeder *et al.*, 1994) can be tested for their participation in tolerance by screening for knock-out mutants. Key enzymes involved in solute accumulation could also be knocked out, such as those involved in proline (Verbruggen *et al.*, 1996; Kiyosue *et al.*, 1996) and betaine biosynthesis (Rathinasabapathi *et al.*, 1997). The recently reported plant Na^+/H^+ anuporter gene is a prime candidate for such gene knock-out testing (Gaxiola *et al.*, 1999; J.M. Pardo, personal communication).

22.11 Halophytes/Xerophytes: the elite signal corps!

Eventually signal components that control downstream events that affect osmotic tolerance phenotype in a significant way will be identified using the model systems. Then the seminal hypothesis that halophytes/xerophytes differ essentially from glycophytes by their exceptional control over the speed and degree to which their perception and can occur, can be examined experimentally. These orthologous signaling components in halophytes/xerophytes should be identifiable by their sequence similarities to counterparts in the model organisms. The halophyte/xerophyte versions of these genes could then be transferred to glycophytes and their effects on responses to stress can be determined. This approach is similar conceptually to the situation where pathogen resistance (R) genes have been identified as components in disease response pathways (Staskawicz, 1995; Rommens *et al.*, 1995). Here R genes from resistant plants have been transferred across species to sensitive genotypes and in some instances resistance has been obtained (Thilmony *et al.*, 1995). The ability to transfer such R genes has, however, been limited by the divergence of other components in the resistance response pathway such that R gene products may be recognized by other signal members of the pathway of only closely related species (Tang *et al.*, 1999).

22.12 Microarrays - the final frontier of stress-controlled genes

The use of various detection techniques such as differential hybridization, subtraction hybridization and RNA display have made it clear that many osmotically induced genes exist in the genomes of plants. The recent development of high-throughput DNA sequencing technologies to complete the sequencing of the entire *Arabidopsis* genome and of large sets of ESTs (expressed sequence tags) representative of all or nearly all expressed genes of several plant species, has opened up the possibility of rapidly knowing essentially all genes that are controlled by any particular environment or developmental parameter, including osmotic stress. Large numbers (several thousand) of separate known nucleotide sequences can be arrayed in a matrix pattern onto membrane filters or glass slides by robotic techniques and subsequently differentially probed with complimentary sequences that have been prepared from stressed and unstressed cells and then labelled with ^{33}P (Service, 1998; Schena *et al.*, 1995). These (essentially very large Northern blots) can then be automatically scanned to determine quantitatively the relative abundance of all transcripts made under the two conditions (stressed and unstressed). For instance this approach has recently been used to characterize the expression patterns of all yeast genes when cells are grown in rich vs. minimal nutrient media (Wodicka, 1997).

One of the most important uses of microarray information will be to determine the specific down-stream or end point genes that are under the control of particular signal pathways through the use of microarray assays on various mutants that are defective in parts of the stress signal transduction system. This kind of microarray assay of gene expression can be expected to reveal unanticipated relationships between different signal components as was recently reported by Holstege *et al.* (1998). Certainly there will be considerable overlap of this control, but the completeness of the microarray approach will allow the identification of the proper combinations of signal components needed to manipulate the activation of the widest array of end-point genes (see *Figure 2*). This information can serve to maximize the probability of having a greater affect on tolerance phenotype through engineering of the appropriate combinations of signal component genes in transgenic plants.

22.13 Bioinformatics - warp speed ahead!

Already the complete sequence of the genomes of 20 microorganisms including the eukaryotic yeast genome is known. The sequencing of 80 additional microorganism genomes is underway and 50 are expected to be finished in 1999. The genome of the worm *Coenorhabditis elegans* is essentially completed and the *Drosophila* genome is moving fast to completion. The entire human genome is scheduled to be sequenced by 2005. The *Arabidopsis* genome was completed in the year 2001 (see Shneider and Smaglick, 1999).

Perhaps you are not impressed by all of the talk about genomics and the revolution to come as a result of the large DNA sequencing projects. To put this in some perspective, think about your computer. Computational power of computers has been doubling every 18 to 24 months for many years now, as evidenced by your need (or at least your

overwhelming urge) to buy a new one at about this rate. There is also a lot of talk about the many wonderful things that computers will be able to do in the future, and you probably have little doubt that the capabilities of computers will continue to expand rapidly. The size of DNA sequence databases is also doubling every 18 to 24 months (Boguski, 1998). Part of the problem in comprehending what this means for the future is that it is difficult to think accurately about something moving so fast. Even though the exact points of landing for these two twin co-supporting events in the life of bioinformatics (increasing DNA sequence database size and increasing computation power) are not easy to predict, some directions can be noticed clearly.

The first rule is that as the database increases, any gene that you find will look more and more like already known sequences, giving you great confidence that your gene functions like these other genes. This gives you important clues and confidence in the kind of experiments that you should do next. Now, if you extend this notion and try to realize the level of the disappointment or satisfaction that you would have in finding that your sequence exactly matched another gene with a very well-established function, you may glimpse the direction of bioinformatics. How much of a match between your gene and one from another species already having a dead-on known function would it take to convince you that the function of your gene was the same (orthologous) and not just related (paralogous) for sure? A perfect match would do it, but what about 50%? Now comes the tricky part. As more sequences and functional knowledge attached (annotated) to these sequences enter the database, your power to predict the function of your new entry increases (Hofmann, 1998; Lake and Moore, 1998). Partial matching of sequences already allows us to make reasonable predictions of function that can then experimentally be tested. Often a gene whose sequence and function is known in a well studied species is used to isolate one of similar sequence in another species. This sequence comparison information is used to isolate orthologous genes with heterologous probes or PCR primers. Several examples of plant genes whose function was predicted and proved by this approach exist including K^+ and PO_4^- transporters (Tang *et al.*, 1995; Muchhal *et al.*, 1996), ATPases (Harper *et al.*, 1994), water channels (Weig *et al.*, 1997; Höfte *et al.*, 1993), protein kinases (Wang *et al.*, 1996), and most recently a Na/H^+ antiporter from *Arabidopsis* (Gaxiola *et al.*, 1999; J.M Pardo, personal communication). The sequence similarity of gene sequences between species is assumed to have resulted from the genes having shared a common ancestral gene (Thornton, 1998). Thus studying this relatedness is a bit like looking at the archeological footprints (common sequences) left by the forerunner gene on its present-day forms.

In the years to come we also will see many overlapping sub-genomes, e.g. the root functioning genes vs. the leaf functioning genes, and yes, the osmotically responding subgenome. Even more importantly, information about interactions between gene products needs to be known and ascribed to each gene (Kanehisa, 1998). We must also concede that information lies beyond the gene sequence that cannot yet be always predicted reliably from the sequence alone, including expression characteristics of the gene and 3-D structural properties of the encoded proteins, so-called proteomics (Brownstein *et al.*, 1998). Conceptually, however, present dogma retains the view that the genetic sequence information is sufficient to build an entire organism when placed in an appropriate (cellular) background (Kaneisha, 1998).

Surely, unexpected hypotheses and inevitably, more and more rules will follow these early adventures into the information content of genomes. But, lest we forget the basic idea, let me end by restating a central principle of bioinformatics. When you find the sequence of a gene and compare it to others, being able to conclude something about that gene (it's a kinase or its product is localized to vascular tissue, etc.) without another experiment, will save you a lot of work and time and let you ponder bigger, better questions. The future of our understanding of genome structure and function as it is related to salt stress tolerance and many other interesting phenotypes is indeed bright!

22.14 References

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